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THE MARINE BIOLOGICAL LABORATORY

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I. TRUSTEES

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II. ACT OF CORPORATION

No. 3170

COMMONWEALTH OF MASSACHUSETTS

Be It Known, That whereas Alpheus Hyatt, William Sanford Stevens, William T. Sedgwick, Edward G. Gardiner, Susan Minns, Charles Sedgwick Minot, Samuel Wells, William G. Farlow, Anna D. Phillips, and B. H. Van Vleck have associated themselves with the intention of forming a Corporation under the name of the Marine Biological Laboratory, for the purpose of establishing and maintaining a laboratory or station for scientific study and investigation, and a school for instruction in biology and natural history, and have complied with the provisions of the statutes of this Commonwealth in such case made and provided, as appears from the certificate of the President, Treasurer, and Trustees of said Corporation, duly approved by the Commissioner of Corporations, and recorded in this office;

Now, therefore, I, HENRY B. PIERCE, Secretary of the Commonwealth of Massachusetts, *do hereby certify* that said A. Hyatt, W. S. Stevens, W. T. Sedgwick, E. G. Gardiner, S. Minns, C. S. Minot, S. Wells, W. G. Farlow, A. D. Phillips, and B. H. Van Vleck, their associates and successors, are legally organized and established as, and are hereby made, an existing Corporation, under the name of the MARINE BIOLOGICAL LAB-

ORATORY, with the powers, rights, and privileges, and subject to the limitations, duties, and restrictions, which by law appertain thereto.

Witness my official signature hereunto subscribed, and the seal of the Commonwealth of Massachusetts hereunto affixed, this twentieth day of March in the year of our Lord One Thousand Eight Hundred and Eighty-Eight.

[SEAL] HENRY B. PIERCE,
Secretary of the Commonwealth

III. BYLAWS OF THE CORPORATION OF THE MARINE BIOLOGICAL LABORATORY

(Revised February 11, 1972)

I. The members of the Corporation shall consist of persons elected by the Board of Trustees.

II. The officers of the Corporation shall consist of a Chairman of the Board of Trustees, President, Director, Treasurer and Clerk.

III. The Annual Meeting of the members shall be held on the Friday following the second Tuesday in August in each year at the Laboratory in Woods Hole, Massachusetts, at 9:30 A.M., and at such meeting the members shall choose by ballot a Treasurer and a Clerk to serve one year, and nine Trustees to serve four years, and shall transact such other business as may properly come before the meeting. Special meetings of the members may be called by the Trustees to be held at such time and place as may be designated.

IV. Twenty-five members shall constitute a quorum at any meeting.

V. Any member in good standing may vote at any meeting, either in person or by proxy duly executed.

VI. Inasmuch as the time and place of the Annual Meeting of members are fixed by these bylaws, no notice of the Annual Meeting need be given. Notice of any special meeting of members, however, shall be given by the Clerk by mailing notice of the time and place and purpose of such meeting, at least (15) days before such meeting, to each member at his or her address as shown on the records of the Corporation.

VII. The Annual Meeting of the Trustees shall be held promptly after the Annual Meeting of the Corporation at the Laboratory in Woods Hole, Massachusetts. Special meetings of the Trustees shall be called by the Chairman, the President, or by any seven Trustees, to be held at such time and place as may be designated, and the Secretary shall give notice thereof by written or printed notice, mailed to each Trustee at his address as shown on the records of the Corporation, at least one (1) week before the meeting. At such special meeting only matters stated in the notice shall be considered. Seven Trustees of those eligible to vote shall constitute a quorum for the transaction of business at any meeting.

VIII. There shall be three groups of Trustees:

(A) Thirty-six Trustees chosen by the Corporation, divided into four classes, each to serve four years. After having served two consecutive terms of four years each, Trustees are ineligible for re-election until a year has elapsed.

(B) Trustees *ex officio*, who shall be the Chairman, the President, the Director, the Treasurer, and the Clerk.

(C) Trustees *Emeriti*, who shall be elected from present or former Trustees by the Corporation. Any member of the Corporation in good standing who has attained the age of seventy years, or has attained the age of sixty-five and has retired from his home institution, and who has served a full elected term as a regular Trustee, shall be designated Trustee Emeritus for life at the next annual meeting provided he signifies his wish to serve the Laboratory in that capacity. Any regular trustee who qualifies for emeritus status shall continue to serve as Trustee until the next Annual Meeting whereupon his office as regular Trustee shall become vacant and be filled by election by the Corporation. The Trustees *ex officio* and *Emeriti* shall have all the rights of the Trustees, except that *Trustees Emeriti* shall not have the right to vote.

The Trustees and officers shall hold their respective offices until their successors are chosen and have qualified in their stead.

IX. The Trustees shall have the control and management of the affairs of the Corporation. They shall elect a Chairman of the Board of Trustees who shall be elected annually and shall serve until his successor is selected and qualified and who shall also preside at meetings of the Corporation. They shall elect a President of the Corporation who shall also be the Vice Chairman of the Board of Trustees and Vice Chairman of meetings of the Corporation, and who shall be elected annually and shall serve until his successor is selected and qualified. They shall appoint a Director of the Laboratory for a term not to exceed five years, provided the term shall not exceed one year if the candidate has attained the age of 65 years prior to the date of the appointment. They may choose such other officers and agents as they may think best. They may fix the compensation and define the duties of all the officers and agents; and may remove them, or any of them except those chosen by the members, at any time. They may fill vacancies occurring in any manner in their own number or in any of the officers. The Board of Trustees shall have the power to choose an Executive Committee from their own number, and to delegate to such Committee such of their own powers as they may deem expedient. They shall from time to time elect members to the Corporation upon such terms and conditions as they may think best.

X. The Associates of the Marine Biological Laboratory shall be an unincorporated group of persons (including associations and corporations) interested in the Laboratory and shall be organized and operated under the general supervision and authority of the Trustees.

XI. The consent of every Trustee shall be necessary to dissolution of the Marine Biological Laboratory. In case of dissolution, the property shall be disposed of in such manner and upon such terms as shall be determined by the affirmative vote of two-thirds of the Board of Trustees.

XII. The account of the Treasurer shall be audited annually by a certified public accountant.

XIII. These bylaws may be altered at any meeting of the Trustees, provided that the notice of such meeting shall state that an alteration of the bylaws will be acted upon.

RESOLUTIONS ADOPTED BY THE TRUSTEES:

I. RESOLVED:

(A) The Executive Committee is hereby designated to consist of not more than ten members, including the *ex officio* members (Chairman of the Board of Trustee, President, Director and Treasurer); and six additional Trustees, two of whom shall be elected by the Board of Trustees each year, to serve for a three-year term. (August 11, 1967).

(B) The Chairman of the Board of Trustees shall act as Chairman of the Executive Committee, and the President as Vice President. A majority of the members of the Executive Committee shall constitute a quorum and a majority of those present at any properly held meeting shall determine its action. It shall meet at such times and places and upon such notice and appoint such sub-committees as the Committee shall determine. (August 12, 1966).

(C) The Executive Committee shall have and may exercise all the powers of the Board during the intervals between meetings of the Board of Trustees except those powers specifically withheld from time to time by the Board or by law. (August 16, 1963).

(D) The Executive Committee shall keep appropriate minutes of its meetings, and its action shall be reported to the Board of Trustees. (August 16, 1963).

II. RESOLVED:

The elected members of the Executive Committee be constituted as a standing "Committee for the Nominations of Officers," responsible for making nominations, at each Annual Meeting of the Corporation, and of the Board of Trustees, for candidates to fill each office as the respective terms of office expire (Chairman of the Board, President, Director, Treasurer, and Clerk). (August 16, 1963).

III. RESOLVED:

Any member of the Corporation in good standing who has attained the age of seventy years, or has attained the age of sixty-five and has retired from his home institution, shall automatically be designated a Life Member of the Corporation provided he signifies his wish to retain his membership in the Corporation. Life Members shall not have the right to vote and shall not be subject to the payment of any dues. (February 16, 1973).

IV. REPORT OF THE DIRECTOR

To: The Trustees of the Marine Biological Laboratory

In the winter just past—the winter of discontent among the Nation's scientists—one of my colleagues, upon hearing me express my concern that I was having to devote too much time to the administrative affairs of the Laboratory and having too little impact on its science, suggested that I read Steven Muller's Gilman Lecture, "The Johns Hopkins Medical Institutions. A Case Study in Administration."

President Muller began with a favorite quote from Malcolm Muggeridge's book, *Affairs of the Heart.* Muggeridge wrote: "I have always been deeply interested in the administrative side of love, which I find more absorbing than its purely erotic aspects. What Lady Chatterley and her game-keeper did in the woods is, to me, of only passing interest, compared with how they got there, what arrangements were made for a shelter in the case of inclement weather, and for refreshments, how they accounted for their absence, whether either party could recover incidental expenses, and if so how? This attitude," he goes on to say, "is, after all, not so unreasonable. Most great generals have admitted that planning campaigns and winning victories in the field is relatively easy compared with arranging transport and supplies. An army, Napoleon said, in one of his most celebrated remarks, marches on its stomach. So do lovers. If the administrative arrangements are faulty, the campaign which follows cannot but be laborious, and even victory brings little satisfaction."

Now I cannot honestly say that I have *always* been deeply interested in the administrative side of either love or science. In fact I hasten to add that I still find little fascination in the administrative side of love. However, increasingly I do find pleasure in seeing the Laboratory operated effectively—which means simply that it continues to provide an environment favoring innovation and communication and minimizing delay and distraction.

What have been the sources of satisfaction in the year just past?

I would mention first the increasing vitality of the Laboratory during the winter months. Quietly the number of year-round principal investigators, who spend all or a major part of their time in Woods Hole, has risen until, with the arrival of E. F. MacNichol, Jr. in the spring of 1973, it has reached an even dozen, with at least one major addition scheduled for October, 1973. Interest is running high, and were it not for the financial problems besetting all of science, we might confidently expect to reach a critical mass by 1975.

Winter teaching is also being expanded. The Boston University Marine Program, which continues to gain scientific (if not financial) strength, has announced a series of twelve short (two-week) intensive undergraduate courses to be offered first in 1973–1974. The faculty will be drawn primarily from Boston University, and will include in addition to the established members of the BUMP faculty, Arthur Echternacht, Lynn Margulis, Robert Jeanne, Barry Cameron and others. Enrollment in these mini-courses is not restricted to Boston University students; it is hoped that they will attract students from many colleges and universities.

For the first time, the Laboratory will offer an undergraduate course during the January short-term (January 7–February 1, 1974). If this experiment is successful, it should lead to the establishment of a substantial January program. The first course will be Developmental Biology, with a faculty including L. E. DeLanney (Instructor-in-Charge), Lester and Lucena Barth, John and Annette Coleman, David Walters and this writer.

The year also brought a perceptible increase in the use of the Laboratory as a Conference Center. I would take special note of two meetings. First, on November 30 and December 1, the Laboratory's Advisory Group on Environmental Programs was convened in Woods Hole. As organized by George Woodwell, the group included F. H. Bormann, A. D. Hasler, K. Mann, R. H. Whittaker, R. Wigley and E. O. Wilson, in addition to a number of colleagues at the Woods Hole Oceanographic Institution, BUMP and the Laboratory's Executive Committee.

The second conference, held on November 4, brought together representatives of the Education Development Center (EDC), the Laboratory, and others interested in increasing the public understanding of science. Eugene Bell convened the meeting in which the group began to explore ways of developing television programs, books and articles and exhibits which might elevate the level of public awareness and understanding of science. Initially the group will concentrate on producing a series of five-minute films for use on commercial television.

We were also reassured by the news that the exceptional quality of our summer courses and training programs will enable all but one of them to proceed in the summer of 1973 without series financial restrictions.

Especially notable is the reorganization of our offerings in marine botany. A Summer Research Program in Experimental Marine Botany, headed by Frank Loewus, will bring together research staff and junior investigators from several institutions, supported by a three-year grant from the National Science Foundation. At the same time Dr. John West will offer for the first time an undergraduate course in Marine Botany. I shall be keenly interested in the response to this undergraduate offering, especially among the colleges and universities of our own Corporation members, in view of the oft-heard arguments in Corporation meetings that we have been neglecting undergraduates.

Despite the "phase-out" of training grants, our Embryology and Physiology courses and Training Program in Fertilization and Gamete Physiology will continue in 1973 without serious cutbacks. No one can predict at this writing what the future will bring, but we must proceed on the assumption that all federal support of these programs will end after this summer.

Unfortunately our Frontiers in Research and Teaching Program, designed to offer advanced training in neurobiology for blacks, was a casualty of the cutback. I say unfortunately because it appeared that the Program was just beginning to take shape and to contribute both to the Laboratory and the Nation. It is difficult to assess why the arguments that won at least temporary restoration of the three aforementioned programs failed to convince the National Institute of Neurological Diseases and Stroke of the importance of the Frontiers Program. Admittedly the quality of the science in the program was not always as high as that in the older-established courses; but I believe progress was being made in meeting the Program's objectives. Apparently the Program failed to gain support either on scientific or social merit.

Three other courses and training programs will continue in 1973 essentially at the 1972 level: the Program in Excitable Membrane Biophysics and Physiology, with gifts from Dr. Ernest B. Wright and IBM; Neurobiology, supported by the Grass Foundation; and Experimental Marine Ecology, funded by the Research Corporation.

I am able to report that the efforts of two special committees culminated in notable successes early in 1973. Robert Allen and the Committee on Brochures, with substantial assistance from Dennis Flanagan, produced two attractive brochures, *The Marine Biological Laboratory* and *A Year-Round Course and Conference Center at the Marine Biological Laboratory*. The recommendations of the Retirement Committee, John Arnold, Chairman, resulted in the appointment of a Consulting Actuary, Mr. Donald Grubbs, and ultimately the adoption on May 1, 1973 of a greatly-improved retirement program for the Laboratory's employees.

In last year's Report I observed that M. R. Carriker would write a summary of the achievements of the Systematics-Ecology Program, which was terminated at the end of August, 1972. Dr. Carriker has now presented the ninth and tenth progress reports and a ten-year summary under the title, *A Decade of Whole Organism Biology*. On September 1, 1973, Dr. Carriker will assume his new responsibilities as professor at the College of Marine Studies of the University of Delaware in Lewes, Delaware.

Finally it is a pleasure to report that the roster of newly elected members of the National Academy of Sciences includes no less than five members of the Corporation of the Laboratory, several of whom are regular summer residents. In addition, one former MBL instructor was singled out for special recognition.

HAROLD CHARLES BOLD is Professor of Botany and Chairman, Division of Biological Sciences, University of Texas. Bold is one of the Nation's leading students of the algae, his comprehensive studies having been collected in a twelve-volume work. He has been unusually influential as a teacher, having received an award as outstanding teacher at the University of Texas, and having directed the advance degree work of over fifty students. At MBL he played an important role in heading the Laboratory's course in marine botany.

KURT JULIUS ISSELBACHER's scientific contributions have primarily been concerned with mammalian metabolism and physiological processes in the liver and the intestine. He is also President-elect of the American Gastro-Enterological Association, a Fellow of the American Academy of Arts and Sciences and recipient of Bengt Ihre Award of the Swedish Medical Society.

AARON BUNSEN LERNER is Professor and Chairman of the Department of Dermatology, Yale University School of Medicine. Lerner is pre-eminent in American dermatology, his stature being derived from his effectiveness as both clinician and as biomedical scientist. Among other achievements, he has isolated two hormones having roles in pigmentation.

In addition to his research accomplishments, Lerner has established an outstanding teaching program, well-balanced in its emphasis on clinical and research dermatology. A number of his students, sharing his interests in biological and clinical science, have also gone on to establish effective dermatology units in other institutions.

PAUL A. MARKS is Professor of Medicine, Columbia University College of Physicians and Surgeons; Dean, Faculty of Medicine, and Vice President in charge of medical affairs, Columbia University. Marks has been among the leaders in clarifying mechanisms of formation of red blood cells and their principal constituent, hemoglobin. He has brought new understanding of the anemias. In this work he has played an important role in the fusion of genetics, molecular biology, and medicine.

William Trager is Professor of Parasitology, Rockefeller University. The Scientific career of Trager, spanning almost 40 years and still actively in progress including summer research at MBL, has included insightful studies of parasitology, symbiosis, tissue culture, and the nutrition of insects and parasitic protozoa. In the course of his career as a teacher and investigator, Trager has published over 100 scientific papers and reviews on such diverse subjects as insect nutrition, insect metamorphosis, intracellular parasitism, the fine-structure of parasitic protozoa, and, in 1970, a noteworthy volume on symbiosis.

Donald D. Brown is a Staff Member, Carnegie Institution of Washington, Department of Embryology, and Professor of Biology at Johns Hopkins. Brown was not only elected to membership, but was also selected by the Academy to receive the U. S. Steel Foundation Award in Molecular Biology, in recognition of "his studies of the structure, regulation, and evolution of genes in animals, particularly the genes specifying ribosomal RNA in *Xenopus* and silk fibroin in *Bombyx*."

In the early 1960's Brown played an important role in the MBL's Embryology course, bringing to it his special insight in molecular mechanisms underlying development.

In extending the Laboratory's congratulations I observed that these additions to the Academy's rolls bring the total number of Academy members in the MBL Corporation to over fifty. This truly remarkable record provides further evidence of the Laboratory's continuing role as one of the Nation's leading centers of research and communication in the life sciences. In fact the three laboratories in Woods Hole provide an uncommonly exciting mix of established investigators and promising young scientists.

A forward look

On May 1, 1973 it appears possible that for the first time in recent years, we shall end the year without a substantial cash deficit. However we should not be misled into thinking that we have found the solution to financial stability. It was only through the combination of the unusual quality and importance of our courses and the devotion, skill, and understanding of our own staff and the program officers at the National Institutes of Health that our training grants were continued in 1973. Moreover it is too early to assess the impact of further restrictions in federal grants to individual investigators. When the laboratory fees were doubled for 1973, I announced that for the summer of 1973 only, no investigator would be denied space because he could not pay the increased rates; upon application, fees would be charged at 1972 rates. At this writing about thirty principal investigators (three representing large groups) have applied for waivers of from ten to fifty per cent of the fees, in an amount totalling about $35,000. It is next to impossible to determine readily the reasons underlying the failure of this group of applicants to obtain sufficient funds. A few are younger scientists, but four-fifths of the "deficit" is attributable to established scientists, some of whom have contributed importantly to the Laboratory for many years.

It seems likely that this list may grow, rather than decrease; the inevitable result will be the retraction of principal investigators into smaller laboratories. *It is essential therefore that we redouble our efforts to attract new investigators of high quality*, for both year-round and summer research.

Concomitantly we must continue our search for new leadership and innovative programs within the existing structure of the Laboratory. Several year-round programs have been discussed; of these, two, developmental ecology and marine genetics, and bioluminescence have begun to take shape. Yet I have observed repeatedly that enthusiasm wanes and progress slows once the protagonists of a proposal leave Woods Hole. Consequently instead of the usual period of up to six months required for the maturation of a major proposal, about fifteen months are required, including two summers, the first for preliminary definition of a problem, the intervening academic year for the circulation of drafts, and a final summer for preparation of a definitive proposal. What we need most is at least one successful "experiment" drawn from the several "models" we have been exploring.

A third large unsolved problem concerns the future of the Library. It is clear that new facilities must be provided by the end of the decade and that they should be developed in cooperation with the other scientific institutions in Woods Hole. However the mechanism whereby a joint facility can be developed most effectively is still unclear. Ideas proposed range from continuing the present arrangement with MBL retaining "ownership" of the Library to the formation of a separate non-profit library corporation, with MBL providing management.

"Where the action is"

In recent years it has become fashionable for directors of laboratories to stress that their organizations are "where the action is," with increasing emphasis on the intersections of technology and society underlying our social concerns. This posture is consonant with the increasing emphasis on contract-supported, directed research and the decline in grants for unfettered basic investigations; it has contributed to today's cry by "management" for the dissolution of the peer review system. According to this view, panels and study sections are composed of self-serving scientists, incapable of assessing the long-range consequences to the Nation of their recommendations. MBL has been proud to say that the contributions of its scientists in basic research have provided the underpinning of many advances in biology and medicine, advances which have had a significant impact in promoting human welfare. I have addressed this question on other occasions. We have not neglected our societal responsibilities. Yet when I hear the expression, "Where the action is," I think not of technology, but of basic science. More than ever the Nation needs centers which will foster basic research of a pioneering nature. It is to that end that we are all working.

1. The Staff

EMBRYOLOGY

I. Instructors

Eric H. Davidson, Associate Professor of Biology, California Institute of Technology, director of course

Gary L. Freeman, Assistant Professor of Biology, University of California San Diego, associate director of course

Joseph Ilan, Associate Professor of Anatomy, Case Western Reserve University

Fotis Kafatos, Professor of Biology, Harvard University

Dennis Smith, Associate Professor of Biology, Purdue University

II. Special Lecturers

Noel de Terra, Institute for Cancer Research, Philadelphia

Lionel I. Rebhun, Department of Biology, University of Virginia

Nina Hillman, Department of Biology, Temple University

III. Assistants

David Miyamoto, Department of Zoology, Duke University
Lenard Evans, Michigan State University

IV. Lectures

E. Davidson	Introduction to the course
G. Freeman	Experimental evidence for the existence of cytoplasmic localizations
E. Davidson	Gene regulation and the localization problem
D. Smith	Temporal aspects of gene regulation during development
J. Ilan	Involvement of t-RNA in the regulation of a specific messenger RNA translation during insect development
N. Verdonk	Activity of the embryo genome in the development of *Lymnaea stagnalis*
	Cytoplasmic localization in the egg of *Bithynia tentaculata*
A. Clement	Development of *Ilyanassa*
J. Collier	Biochemical aspects of the development of *Ilyanassa*
J. Arnold	Role of egg cortex in the development of squid
T. Humphreys	Regulation of RNA synthesis during sea urchin development
P. Gross	Biochemical studies in sea urchin differentiation
J. Ilan	Specification of the initiation factors for protein synthesis during insect development
R. Quatrano	Steps leading to the formation of the rhizoid cells in *Fucus*
R. C. King	Genetic studies of oogenesis in insects
Klaus Sander	Localization of the factors involved in the establishment of segmental order in insects

PHYSIOLOGY

I. Consultants

Andrew G. Szent-Györgyi, Professor of Biology, Brandeis University

II. Instructors

John J. Cebra, Professor of Biology, Johns Hopkins University, in charge of course
Anthony C. Allison, Head, Cell Pathology Division, Medical Research Council Clinical Research Centre, Harrow, England
L. William Clem, Professor of Immunology and Medical Microbiology, University of Florida School of Medicine
Richard E. Ecker, Associate Biologist, Argonne National Laboratories
Brian J. McCarthy, Professor of Biochemistry, University of Washington
Robert A. Prendergast, Associate Professor of Ophthalmology, Johns Hopkins University School of Medicine
David A. Yphantis, Professor of Biochemistry and Biophysics, University of Connecticut

III. Special Lecturers

Leon Weiss, Professor of Anatomy, Johns Hopkins University School of Medicine
Shraga Segal, Postdoctoral fellow, Department of Biology, Johns Hopkins University and Instructor, Weizmann Institute of Science, Israel

Emil Unanue, Associate Professor of Pathology, Harvard Medical School
Susan Lowey, Asst. Professor of Biological Chemistry, Children's Cancer Research foundation
Hugh Huxley, Cambridge University
Thomas Pollard, Asst. Professor of Anatomy, Harvard Medical School
Andrew Szent-Györgyi, Professor of Biology, Brandeis University
Shinya Inoué, University of Pennsylvania
Leonard Herzenberg, Professor of Genetics, Stanford University School of Medicine
Raymond Stephens, Brandeis University
Richard Weisenberg, Temple University
Y. C. Lee, Associate Professor of Biology, Johns Hopkins University
Bruce Merrifield, Professor of Biochemistry, Rockefeller University
Moselio Schaechter, Professor of Molecular Biology and Microbiology, Tufts University Medical School
David Benjamin, Asst. Professor of Microbiology, University of Virginia School of Medicine
Francis Carlson, Professor of Biophysics, Johns Hopkins University
K. E. Van Holde, Professor of Biophysics, Oregon State University
Michael Edidin, Associate Professor of Biology, Johns Hopkins University
Renata Cathou, Asst. Professor of Biochemistry, Tufts University School of Medicine
Christopher Cordle, Graduate Student, Department of Biology, Johns Hopkins University
Gerald Weissmann, Professor of Medicine, New York University School of Medicine
Werner Loewenstein, Professor of Physiology, University of Miami Medical School
Elvin Kabat, Professor of Microbiology, Columbia University College of Physicians and Surgeons

IV. Staff Associates

Dennis Barrett, University of California at Davis
Dennis Powers, State University of New York at Stony Brook
Robley Williams, Yale University

V. Course Assistants

Martha Barcalow Barrett, University of California at Davis
Calvina Baumgartner, Johns Hopkins University
Marvin Cuchens, University of Florida College of Medicine
Carlton Paul, University of Connecticut
David Yocum, University of Florida College of Medicine

VI. Assistant Consultants/Teachers

Susan Craig, Johns Hopkins University
Patricia Johes, Johns Hopkins University
Robert Reese, Johns Hopkins University
Daniel Tracey, Johns Hopkins University

VII. Lectures

D. Yphantis	Physical biochemistry I Physical biochemistry II Physical biochemistry III

R. Williams	Subunit association and cooperativity in proteins, I
	Subunit association and cooperativity in proteins, II
R. Ecker	Growth and protein synthesis in bacterial populations and individual cells
	Kinetics of protein synthesis in amphibian oocytes and early embryos
	The nature and fate of proteins synthesized in early amphibian development
	The kinetics of protein synthesis in sea urchin and mammalian embryos
D. Powers	Biochemical foundations for protein polymorphisms in natural populations
R. Prendergast	The immune response
J. Cebra	Antibodies and their reactions with antigens
	Antigenic determinants as probes of antibody sites
R. Prendergast	Cellular immunity
W. Clem	Ontogeny and phylogeny of the immune response
L. Weiss	Cells and tissues of the immune response
S. Segal	Cellular interactions in the immune response
B. McCarthy	Structure of interphase chromosomes
	Fractionation of chromatin—background and approaches
	Properties of fractionated chromatin
	The specificity of DNA/RNA hybridization reactions
	Unique sequence DNA/RNA hybridization in eukaryotes
A. Allison	Tolerance, unresponsiveness, and controls of the immune response
R. Prendergast	Immunopathology with special reference to immune-complex disorders
W. Clem	Immunoglobulin polymorphism and the secondary biologic activities of antibodies
J. Cebra	Antibody structure
	Genetic control of immunoglobulin—biosynthesis and generation of diversity
E. Unanue	The role of macrophage in the immune response
S. Lowey	Chemistry of contractile systems
H. Huxley	Basic structure of conventional muscle
A. Allison	Microfilaments and microtubules in cell biology
T. Pollard	Molecular mechanisms of cell motility
A. Szent-Györgyi	Regulatory systems in muscle
S. Inoué	The dynamics of the mitotic spindle
L. Herzenberg	Fluorescence-activated electronic cell sorting
R. Stephens	Biochemical aspects of the microtubular systems
A. Allison	Cellular contractile systems in relation to endocytosis and discharge of packaged receptors
R. Weisenberg	Organization of microtubular protein polymerization
D. Barrett	Proteins of the early sea urchin embryo—interface between physiology and development
D. Powers and J. Cebra	Purification and characterization of proteins and peptides
Y. Lee	Glycoproteins—structure of oligosaccharides bound to proteins
J. Cebra	Modification and cleavage of proteins—strategy for sequencing

D. Powers	Sequential degradations of polypeptides, end group analysis, and prosthetic groups
B. Merrifield	Stepwise polypeptide synthesis
M. Schaechter	Organization of bacterial nucleus
D. Benjamin	Structural basis for the termination of immunologic tolerance
F. Carlson	Quasi-elastic photon scattering—a technique for studying molecular movement and biologic motility
K. Van Holde	Electric dichroism of DNA and chromatin
M. Edidin	Surface antigens in the study of membrane structure
R. Cathou	Circular dichroism and biomolecular conformation Fluorescence spectra of biological molecules
C. Cordle	Nanosecond fluorometry—measurement and application
G. Weissmann	Liposomes as membrane models in immunopathology
W. Loewenstein	Cellular communication: growth and differentiation
E. Kabat	Attempts at construction of a three dimensional model of the polypeptide backbone of variable region of immunoglobulin light chain

EXPERIMENTAL MARINE BOTANY

I. Consultants

Harlyn O. Halvorson, Brandeis University
H. W. Siegelman, Brookhaven National Laboratory
W. Randolph Taylor, University of Michigan

II. Instructors

Frank A. Loewus, Professor of Biology, State University of New York at Buffalo, in charge of course
Johan A. Hellebust, Associate Professor of Botany, University of Toronto
Ralph S. Quatrano, Associate Professor of Botany, Oregon State University
Jerome A. Schiff, Professor of Biology, Brandeis University
Michael J. Wynne, Associate Professor of Botany, University of Texas at Austin

III. Special Lecturers

Nina Allen, Research Associate, State University of New York at Albany
Winslow Briggs, Professor of Biology, Harvard University
R. Bachofen, Professor, University of Zurich, Switzerland
David Chapman, Associate Professor of Biology, University of Chicago
Hans Gaffron, Professor, Florida State University
Martin Gibbs, Professor of Biology, Brandeis University
Andre Jagen Dorf, Professor of Botany, Cornell University
Peter Kilham, Assistant Professor of Zoology, University of Michigan
Norman Krinsky, Professor, Tufts University, School of Medicine
Carl A. Price, Professor of Biochemistry, Rutgers University
Joe Ramus, Assistant Professor of Botany, Yale University
Anthony G. San Pietro, Professor of Botany, Indiana University

IV. Assistants

Monica Tsang, Brandeis University
Richard Wetherbee, University of Michigan

V. Lectures

Michael J. Wynne	Introduction to the benthic marine algae: the Chlorophyta
	Introduction to the benthic marine algae: the Phaeophyta and Rhodophyta
	The benthic algae as research tools
Peter Kilham	Ecological physiology of plantonic marine diatoms
Johan A. Hellebust	Transport systems for organic substrates in marine diatoms
	Effects of salinity on algal metabolism
	Excretion of organic compounds by algae
Jerome A. Schiff	Pathways of sulfate reduction and utilization in algae
David Chapman	Carotenoids: biosynthesis and function in photosynthetic systems
Jerome A. Schiff	Plastid development and evolution
	Plastid development and inheritance
	Evolution of photosynthetic pigment systems
Carl A. Price	Isolation of algal cells and sub-cellular organelles by centrifugation
Anthony G. San Pietro	Photosynthetic electron transport and photophosphorylation I.
	Photosynthetic electron transport and photophosphorylation II.
	Photosynthetic electron transport and photophosphorylation III.
Hans Gaffron	Photochemical cooperation of dissimilar pigments in the chloroplast
	Photochemical electron transport and hydrogen evolution in green algae and purple bacteria in the absence of synthetic reactions
Ralph S. Quatrano	Marine algae in developmental biology
	Cytoplasmic polarity: a mechanism of cellular differentiation in *Fucus* and *Acetabularia*
Norman Krinsky	Function of carotinoid pigments
Winslow Briggs	Biochemistry of phytochrome: a light-sensitive regulatory protein in plants
Frank A. Loewus	Carbohydrate interconversions involving inositol
	Carbohydrate exudates of plant origin and their functional aspects
Martin Gibbs	Photorespiration
Nina Allen	Motility of chloroplasts in algae
Joe Ramus	Extracellular polysaccharide production by red algae
Andre Jagendorf	Chemiosmotic concepts in photophosphorylation
R. Bachofen	Transitions in the ATP levels in *Chlorella fusca* and *Micrococcus denitrificians* due to environmental change

EXPERIMENTAL INVERTEBRATE ZOOLOGY

I. Consultants

Frank A. Brown, Jr., Northwestern University
James F. Case, University of California, Santa Barbara
C. Ladd Prosser, University of Illinois

Clark P. Read, Rice University
A. C. Redfield, Woods Hole Oceanographic Institution
W. D. Russell-Hunter, Syracuse University

II. Instructors

Robert K. Josephson, University of California, Irvine, in charge of course
A. Gelperin, Princeton University
M. Greenberg, Florida State University
J. B. Jennings, University of Leeds
R. Johnson, University of Chicago
C. Mangum, College of William and Mary
J. Morin, University of California, Los Angeles
T. Schopf, University of Chicago
S. Vogel, Duke University

III. Special Lecturers

A. Farmanfarmaian, Rutgers University
A. Haschemeyer, Hunter College
G. Holz, Upstate Medical Center
M. Mote, Temple University
N. Rushforth, Case Western Reserve University
D. Mellon, University of Virginia

IV. Assistants

J. Cornell, University of California, Berkeley
K. Flessa, State University of New York at Stony Brook

V. Lectures

R. Josephson	Introduction to the course
S. Vogel	Porifera
R. Josephson	Overview of animal phyla
	Cnidaria and Ctenophora
R. Johnson	Introduction to field studies
C. Mangum	Annelida
T. Schopf	Lophophorates
A. Gelperin	Arthropoda
M. Greenberg	Mollusca I.
	Mollusca II.
J. Morin	Echinodermata
	Protochordates
S. Vogel	Fluids and animals I: boundary layers and how they grow
	Fluids and animals II: flow and adaptations of sessile animals
	Passive ventilation
C. Mangum	Respiration I. exchange
	Respiration II. transport
M. Greenberg	Patterns of circulation in invertebrates
C. Mangum	Temperature adaptation

T. Schopf	Population genetics of marine organisms
	Specialization, speciation and distribution
J. Jennings	Symbiotic relations in platyhelminthes
	Digestive physiology of Turbellaria
R. Josephson	On the origin of nervous systems
A. Gelperin	Command and executive neurons
	Neuronal oscillators and rhythmic behavior
	Regulation of feeding
J. Morin	Colonial organization and coordination
	Bioluminescence in coelenterates
R. Josephson	The design of striated muscle
M. Greenberg	Hearts and visceral muscle
	Comparative neuropharmacology

MARINE ECOLOGY

I. Consultants

J. Woodland Hastings, Harvard University
Howard L. Sanders, Woods Hole Oceanographic Institution
Lawrence B. Slobodkin, State University of New York at Stony Brook
Roger Y. Stanier, Institute Pasteur, Paris

II. Instructors

Holger W. Jannasch, Senior Scientist, Woods Hole Oceanographic Institution, in charge of course
Edward R. Leadbetter, Professor of Microbiology, Amherst College
Ralph Mitchell, Professor of Applied Microbiology, Harvard University
Kenneth H. Nealson, Assistant Professor of Microbiology, University of Massachusetts
Ralph S. Wolfe, Professor of Microbiology, University of Illinois at Urbana
Theodore J. Smayda, Professor of Marine Biology, University of Rhode Island
Edward O. Wilson, Professor of Zoology, Harvard University
J. Frederick Grassle, Assistant Scientist, Woods Hole Oceanographic Institution

III. Special Lecturers

Jelle Atema, Assistant Scientist, Woods Hole Oceanographic Institution
Bruno Battaglia, Professor, University of Padua, Italy
Ercole Canale-Parola, Associate Professor, University of Massachusetts
Robert L. Guillard, Associate Scientist, Woods Hole Oceanographic Institution
Galen E. Jones, Professor, University of New Hampshire
R. E. Kallio, Professor, University of Illinois at Urbana
John H. Kanwisher, Senior Scientist, Woods Hole Oceanographic Institution
Eugene P. Odum, Professor, University of Georgia
Luigi Provasoli, Professor, Yale University
John H. Ryther, Senior Scientist, Woods Hole Oceanographic Institution
Howard L. Sanders, Senior Scientist, Woods Hole Oceanographic Institution
Larry B. Slobodkin, Professor, State University of New York at Stony Brook
John Todd, Assistant Scientist, Woods Hole Oceanographic Institution
Wolf Vishniac, Professor, University of Rochester
Meyer J. Wolin, Professor, University of Illinois at Urbana

IV. Research Associates

Anatol Eberhard, Associate Professor, Ithaca College
Alex Keynan, Professor, The Hebrew University at Jerusalem

V. Assistants

William H. Brakel, Yale University

VI. Lectures

Holger W. Jannasch	Introduction to the course
	Marine microbiology, I and II
	Experimental microbial ecology, I and II
	Deep sea microbiology
Ralph S. Wolfe	Introduction to microbial ecology
	Life without air
	Anaerobic metabolic food chains
	Microbial formation of methane
	Aerobic ways of making a living
	Higher bacteria and their ecological distribution
	Microbial sulfur cycle
Kenneth H. Nealson	Bacterial genetics, I and II
	Bioluminescence, general
	Bacterial bioluminescence
Edward R. Leadbetter	Microbial symbiosis
Ralph Mitchell	Intermicrobial predation
	Bacterial chemotaxis
	Microbial ecology of surfaces
	Ecological approaches to water pollution
Theodore J. Smayda	Components of phytoplankton
	Sargassum community
	Global pattern in phytoplankton distribution
	Nutrition of phytoplankton
	Diurnal rhythms in phytoplankton
	Phytoplankton enrichments
J. Frederick Grassle	Benthic communities
	Control of population size
	Genetic variation and community diversity
	Coral reefs
Edward O. Wilson	Theoretical and experimental zoogeography, I and II
	Sociobiology, I and II
	Chemical communication I
Jelle Atema	Chemical communication II
Bruno Battaglia	Ecological genetics of marine animals
Ercole Canale-Parola	Ecology of Spirochaetes
Robert L. Guillard	Physiological clones of phytoplankton
Galen E. Jones	Fractionation of stable sulfur isotopes in the environment
R. E. Kallio	Hydrocarbon oxidation, environmental and geological aspects
	Biochemistry of hydrocarbon degradation
John H. Kanwisher	Physiology and the ecological dogma

Eugene P. Odum	Energy-based inventory; a foundation for land use management
	Is out-welling a major factor in coastal productivity?
Luigi Provasoli	Interactions between algae and bacteria
	Vitamin cycling in the sea
John H. Ryther	Aquaculture
Howard L. Sanders	Diversity in marine benthic communities
	Deep sea benthic ecology
Larry B. Slobodkin	Evolutionary strategy
John Todd	Introduction into environmental ethology
Wolf Vishniac	Productivity of the ocean
	Antarctic microbiology
	Environmental conditions for the origin of life

NEUROBIOLOGY

I. Instructors

Michael V. L. Bennett, Professor of Anatomy, Albert Einstein College of Medicine, Yeshiva University

John E. Dowling, Professor of Biology, Harvard University

Anthony L. F. Gorman, Professor of Physiology, Boston University School of Medicine

Rodolfo R. Llinas, Professor of Physiology and Biophysics, University of Iowa

George Pappas, Professor of Anatomy, Albert Einstein College of Medicine, Yeshiva University

Victor P. Whittaker, Sir William Dunn Reader in Biochemistry, Cambridge University

II. Special Lecturers

S. G. Waxman, Albert Einstein College of Medicine
F. S. Werblin, University of California, Berkeley
B. B. Boycott, Kings College London
George Katz, Columbia University
Maurizio Mirolli, National Institute of Mental Health
D. O. Carpenter, National Institutes of Health
H. Gainer, National Institutes of Health
H. Ripps, New York University School of Medicine
J. E. Brown, Vanderbilt University School of Medicine
M. H. Goldstein, John Hopkins University School of Medicine
A. B. Steinbach, University of California at Berkeley
F. C. G. Hoskin, Illinois Institute of Technology
M. J. Dowdall, Cambridge University
F. Dodge, Rockfeller University
J. G. Nicholls, Harvard Medical School

III. Assistants

Miss Carol O'Connor, Boston University

IV. Lectures

M. V. L. Bennett	Central dogma I: impulse conduction
	Central dogma II: synaptic transmission

G. D. Pappas	Membranes and fine structure of neurons
S. G. Waxman	Neurobiology of axonal specializations
G. D. Pappas	The fine structure of chemical synapses in relation to function
F. S. Werblin	Functional organization of the vertebrate retina
G. D. Pappas	The fine structure of electrical synapses in relation to function
B. B. Boycott	Approaches to the neural mechanisms of behavior: an appraisal
A. L. F. Gorman	Membrane theory: properties of nerve membrane
	Membrane potential of nerve and glial cells and diffusion through the extracellular space
George Katz	Introduction to electrophysiological measurements
Maurizio Mirolli	Geometrical factors determining the electrotonic properties of nerve cells
A. L. F. Gorman	Metabolism of nerve cells: Na-K exchange pump and electrogenic effects
D. O. Carpenter	The state of ions in water inside nerve cells
H. Gainer	Protein synthesis in identifiable nerve cells
A. L. F. Gorman	Specialized regions of nerve cells: synaptic and sensory membrane
J. E. Dowling	Review: anatomy, chemistry and physiology of photoreceptors
H. Ripps	Energy, quanta, and vision
J. E. Brown	Receptor potentials and adaptation in the *Limulus* ventral eye
J. E. Dowling	The processing of visual information
M. H. Goldstein	The physiology of cochlear hair cells and lateral line organs
	Physiology of the sensory cortex
A. B. Steinbach	General aspects of synaptic transmission at neuromuscular junctions
	Postsynaptic receptor functions during synaptic transmission
R. Llinas	Transmission at the squid giant synapse
	Depolarization-release coupling at the squid giant synapse
M. V. L. Bennett	Electric organs: how the diversity of nature instructs and aids the experimenter
	Electroreceptors as models of sensitive synapses, or certain fish feel very small voltages that we probably could hear
	Electrotonic synapses: physiology and function
	Chemical *vs.* electrical transmission, or why send a letter when you can telephone?
J. G. Nicholls	The leech, an animal you can get attached to
V. P. Whittaker and M. J. Dowdall	Introduction to the course
V. P. Whittaker	Subcellular fractionation of nerve tissue: methods and results
	Synaptosomes as miniature cells
	Compartmentation of transmitters
F. C. G. Hoskin	Enzyme inhibitors as neurochemical tools
M. J. Dowdall	Cholinergic systems: comparative aspects
D. Soifer	Microtubular function in relation to axonal transport

M. V. L. Bennett	Interpretation of CNS recordings, or beauty is in the eye of the oscilloscope
F. Dodge	Climbing the dendritic tree
M. V. L. Bennett	More on CNS recording The case of the hatchet fish giant synapse: a missing soma is found but mysteries remain
R. Llinas	Field potential analysis of cerebellar circuits Functional properties of the climbing fiber—purkinje cell system Function properties of the mossy fiber—granule cell—Purkinje cell system

THE LABORATORY STAFF

Homer P. Smith, General Manager

Miss Jane Fessenden, Librarian
John J. Valois, Manager, Supply Department
Lewis M. Lawday, Assistant Manager, Supply Department
Frank A. Wildes, Controller
Robert Kahler, Superintendent, Buildings and Grounds
Robert Gunning, Assistant Superintendent, Buildings and Grounds
Jim A. Hancock, Manager, Department of Research Service

GENERAL OFFICE

Edward J. Bender
Miss Christina Bowden
Mrs. Florence C. Butz
Mrs. Shirley J. Delisle
Mrs. Joyce C. Glass
Mrs. Vivian I. Manson
Miss Elaine C. Perry
Mrs. Lorraine A. Ruddick
Miss Mary Tavares

LIBRARY

Mrs. Virginia Brandenburg
David J. Fitzgerald
Miss Katherine H. Glynn
Mrs. Lenora Joseph
Miss Holly Karalekas
Anthony J. Mahler

MAINTENANCE OF BUILDINGS AND GROUNDS

Eldon P. Allen
Lee Bourgoin
Bernard F. Cavanaugh
Robert T. Chase
James S. Clarke
John V. Day
Manuel P. Dutra
Glenn R. Enos
Charles K. Fuglister
Mrs. Elizabeth J. Geggatt
Richard E. Geggatt, Jr.
Mrs. Elizabeth Kuil
Donald B. Lehy
Ralph H. Lewis
Russell F. Lewis
Wilson A. Little, Jr.
William M. Lochhead
Alan G. Lunn
Stephen Mills
Mrs. Lillian A. Stetson
Frederick E. Thrasher
Frederick E. Ward
Ralph D. Whitman

DEPARTMENT OF RESEARCH SERVICE

Gail Cavanaugh
Lowell V. Martin
Miss Christine A. Simmons
Frank E. Sylvia

SUPPLY DEPARTMENT

Jeffrey Balmer
Edward Enos
Philip C. Engles
Clifford A. Goudey
David H. Graham
Robert O. Lehy
Miss Joyce B. Lima
A. Dickson Smith
Eugene A. Tassinari
Bruno F. Trapasso
John M. Varao

2. Investigators, Lillie, Grass, and Rand Fellows; Students

Independent Investigators, 1972

Ache, Barry W., Assistant Professor, Florida Atlantic University
Adelman, William J., Chief, National Institute of Neurological Diseases and Blindness
Agranoff, Bernard W., Professor of Biological Chemistry and Research Biochemist, University of Michigan
Albuquerque, Edson X., Associate Professor of Pharmacology, State University of New York at Buffalo
Allen, Robert Day, Professor and Chairman, State University of New York at Albany
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Tsang, Monica Lik-Shing, Brandeis University
Tucker, Gail S., University of Kansas
Turner, Pamela Fay, Illinois Institute of Technology

Van Mourik, Catharina H. E., Oakland University

Wang, Jane, University of Connecticut
Watts, John Albert, Jr., University of Maryland
Weatherly, W. Paul, Yale University
Wishner, Karen, University of Chicago
Wood, Gary, State University of New York at Albany
Wood, James, University of Michigan

Yocum, David Eugene, University of Florida
Yosha, Sandra, Indiana University
Youngdahl, Pamela E., University of Oregon
Yphantis, Sandra, Physiology Course, Marine Biological Laboratory
Yulo, Theresa, University of Rochester Medical School

Zakevicius, Jane M., New York University Medical Center
Zeitler, Rodney, University of Iowa

Library Readers, 1972

Allen, Garland E., Assistant Professor of Biology, Washington University
Anderson, Rubert S., Independent Library Reader, Marine Biological Laboratory

Bang, F. B., Professor, Johns Hopkins University
Benjamin, Thomas L., Associate, Department of Viral Oncology, Public Health Research Institute of City of New York, Inc.
Berridge, Michael John, Senior Scientific Officer, University of Cambridge, England
Bridgman, Anna Josephine, Professor of Biology, Agnes Scott College
Burbanck, W. D., Professor of Biology, Emory University

Carlson, Francis D., Professor, Johns Hopkins University
Cassidy, Fr. Joseph D., Assistant Professor of Biology, University of Notre Dame
Clement, Anthony C., Professor of Biology, Emory University
Cobb, Jewel Plummer, Dean of the College and Professor of Zoology, Connecticut College
Cohen, Seymour S., Professor of Microbiology, University of Colorado Medical Center
Copeland, Donald Eugene, Professor of Biology, Tulane University
Couch, Ernest F., Assistant Professor of Biology, Texas Christian University

Davis, Bernard D., Professor, Harvard Medical School
DeLanney, Louis E., Professor of Biology and Chairman, Ithaca College

Eder, Howard A., Professor of Medicine, Albert Einstein College of Medicine
Eisen, Herman H., Professor and Head, Department of Microbiology, Washington University

Fussell, Catherine P., Assistant Professor of Biology, The Pennsylvania State University

Gabriel, Mordecai L., Dean, School of Science, Brooklyn College
German, James L., III, Investigator; Director, Laboratory of Human Genetics, The New York Blood Center
Ginsberg, Harold S., Professor and Chairman, Department of Microbiology, University of Pennsylvania
Green, James W., Professor of Physiology, Rutgers University

Handler, Philip, President, National Academy of Sciences
Hillman, Nina, Research Associate, Temple University
Hillman, Peter, Guest Professor, The Hebrew University of Jerusalem
Hillman, Ralph, Professor, Temple University
Holtzman, Eric, Associate Professor of Biological Sciences, Columbia University

Kempton, Rudolf, Professor Emeritus of Biology, Vassar College
Keosian, John, Independent Library Reader, Marine Biological Laboratory
Keynan, Alex, Vice President, Hebrew University of Jerusalem
Kirschenbaum, Donald M., Associate Professor, Downstate Medical Center, State University of New York.
Krassner, Stuart, Associate Professor, University of California, Irvine
Kravitz, Edward A., Professor of Neurobiology, Harvard Medical School
Krinsky, Norman I., Professor of Biocemistry and Pharmacology, Tufts University School of Medicine
Kuffler, Stephen W., Robert Winthrop Professor of Neurobiology and Department Chairman, Harvard Medical School

Laderman, Aimlee D., Instructor, Ramapo College of New Jersey
Lash, James W., Professor of Anatomy, University of Pennsylvania School of Medicine
Leighton, Joseph, Professor and Chairman, Department of Pathology, The Medical College of Pennsylvania
Lerner, Aaron B., Professor of Dermatology, Yale University
Luria, S. E., Professor of Biology, Massachusetts Institute of Technology

MacDonald, Eve L., Assistant Professor, Wilson College
Marks, Paul A., Dean and Vice President in Charge of Medical Affairs, Columbia University College of Physicians and Surgeons
Marsland, Douglas, Research Professor Emeritus Biology, New York University
Mizell, Merle, Professor of Biology, Tulane University
Morrell, Frank, Professor of Neurology, New York Medical College

Oschman, James L., Assistant Professor, Northwestern University

Pachas, Willy N., Chief, Arthritis and Rheumatism Section, Veterans Administration Hospital

Reiner, John M., Research Professor of Pathology, Professor of Biochemistry, Albany Medical College of Union University
Roth, Jay S., Professor of Biochemistry, University of Connecticut
Rowland, Lewis P., Professor and Chairman, Department of Neurology, University of Pennsylvania
Rosenberg, Evelyn Kivy, Associate Professor, Jersey City State College
Roy, Guy, Assistant Professor, University of Montreal
Rubinow, Sol I., Professor of Biomathematics, Cornell University Medical College
Rugh, Roberts, Research Biologist, Health, Education and Welfare

Scott, George Taylor, Professor of Biology, Oberlin College
Shemin, David, Professor of Biochemistry, Northwestern University
Sohal, Rajindar S., Associate Professor of Biology, Southern Methodist University
Sonnenblick, B. P., Professor of Zoology, Rutgers—The State University
Steinberg, Malcolm S., Professor of Biology, Princeton University
Strittmatter, Philipp, Professor and Head, Department of Biochemistry, University of Connecticut Health Center

Tanzer, Marvin L., Associate Professor, University of Connecticut Health Center
Terebey, Nicholas, Fellow, State University of New York, Downstate Medical Center
Thomas, Lewis, Dean, Yale University School of Medicine
Tweedell, Kenyon S., Professor of Biology, University of Notre Dame

Wainio, Walter, Professor and Chairman, Department of Biochemistry, Rutgers College—The State University of New Jersey
Waksman, Byron H., Professor of Microbiology, Yale University
Weiss, Leon, Professor of Anatomy, Johns Hopkins University
Wichterman, Ralph, Professor of Biology, Temple University
Wilkie, D. R., Jodrell Professor of Physiology, University College, London, England
Wilson, Thomas Hastings, Professor of Physiology, Harvard Medical School
Wittenberg, Jonathan B., Professor of Physiology, Albert Einstein College of Medicine
Wheeler, George E., Professor of Biology, Brooklyn College
Woolpy, Jerome H., Associate Professor of Biology, Earlham College

Yntema, Chester L., Professor of Anatomy, Upstate Medical Center, State University of New York
Young, Janice E., Assistant Professor of Biology, Keuka College

Students, 1971

All students listed completed the formal course program. Asterisk indicates students completing post-course research program.

ECOLOGY

Ament, Alison S., University of Pennsylvania
Brostoff, William N., University of California, Irvine
*Cohen, Robert M., Amherst College
*Cronan, Christopher S., University of Pennsylvania
*Dieterich, Ann, Reed College
*Gill, Mary L., University of Massachusetts
Goodman, Jeffrey H., Harvard University
Hall, John G., University of Connecticut
*Laws, A. Charles, Harvard University
*Magner, James A., University of Illinois
Moore, Carol A., Montclair State College
*Patt, Tom E., University of Wisconsin
Raghaven, Chitra, University of Massachusetts
*Romesser, James A., University of Illinois
Tingle, Marjorie A., Brandeis University
Weisbrod, Roberta E., New York Blood Center
Yingst, Josephine Y., University of Southern California

EMBRYOLOGY

*Amenson, Christopher S., California Institute of Technology
*Boyer, Barbara C., University of Iowa
*Brandis, John W., Indiana University
*Burnside, Mary B., Harvard Medical School
*Carroll, Alan G., Michigan State University
*Daniel, Jon C., University of Pennsylvania
*Donohoo, Patricia K., Cornell University
*Fischer, Albrecht E., University of Cologne
*Frankel, Fred Robert, University of Pennsylvania
*Hollinger, Thomas G., Purdue University
*Honig, Barry H., Columbia University
Honjo, Tasuku, Carnegie Institution of Washington

*Kozak, Christine A., Yale University
*Margolis, Robert L., Wesleyan University
*Nadel, Marion Ruth, Harvard University
*Palatnik, Carl M., State University of New York at Stony Brook
*Rose, George, Oregon State University
Trischmann, Thomas M., Johns Hopkins University
*Vournakis, John N., Massachusetts Institute of Technology

EXPERIMENTAL BOTANY

*Cottet, Gail L., Keuka College
*Crayton Michele A., Oregon State University
*Goldman, Mark E., University of Massachusetts at Boston
*Harris, Gary C., University of Massachusetts
Krohn, Joyce Levingston, State University of New York at Stony Brook
Rodriguez, Kyrsis R., University of Puerto Rico
Sakai, Ann K., Oberlin College
*Staley, Jonathan H., University of Massachusetts
Story, Judith A., University of New Hampshire
*Telzer, Bruce R., Yale University
*Tripp, Martha J., Florida State University
Vaisberg, Abraham J., Brandeis University
*Wilson, Ernest, Virginia State College
Yagoda, Janet M., University of Massachusetts at Boston

PHYSIOLOGY

Britz, Judith, Goucher College
Britz, Steven, Harvard University
Brown, Rodney, I.B.M.
*Brunhouse, Robert, Johns Hopkins University
*Cartwright, T. Joiner, University of Hawaii
*Chaires, Jonathan, University of California, Santa Cruz
*Chavez, Lloyd, University of Virginia Medical School
*Craane, Julius, De Paul University
De Francesco, Laura, University of California, San Diego
*Devreotes, Peter, Johns Hopkins University
*Edwards, Benjamin, University of California, Davis
*Ettienne, Earl, Oakland University
Funkhouser, Edward, Rutgers University
*Gaydos, Margaret, University of Virginia
Greene, Milford, Wesleyan University
Haseman, Diane, University of Missouri
Kessler, David, Amherst University
*Kuczmarshi, Edward, Yale University
*Lamos, Candace, Michigan State University
*Langford, George, University of Pennsylvania
*Laue, Thomas, University of Virginia Medical School
*LeBoeuf, Renee, Oregon State University
*Levine, Robert, Harvard University
*Lobut, Aline, Institute of Molecular Biology, Paris
*Marshak, Ann, Washington University, St. Louis
*Mounts, Phoebe, University of Pittsburgh
Naidorf, Kenneth, Columbia University, College of Physicians and Surgeons
*Odell, Garrett, Rensselear Polytechnic Institute
Prebluda, Jeffrey, Johns Hopkins University
*Tanaka, Richard, University of Southern California
Weaver, Terry, Ohio State University

INVERTEBRATE ZOOLOGY

Aspey, Wayne P., Ohio University
*Battles, Donald R., College of William and Mary
Bell, David M., Princeton University
Berry, Carol Dawn, Goddard College
Bloedel, Carla, Vanderbilt University School of Medicine
Brechnock, Edward L., University of Hawaii
Cintron, Nitza M., University of Puerto Rico
Cohen, Larry, Yale University School of Medicine
*Costello, Elaine, Drew University
Dehn, Paula F., De Paul University
*DeNiro, Michael, J., University of West Indies
Ely, Carol, Wilson College
Factor, Jan Robert, Brooklyn College
Fairchild, Lincoln, University of Massachusetts at Boston
*Forsythe, Dave, Princeton University
Friesen, Wolfgang Otto, University of California, San Diego
*Gunter, Pamela, Spelman College
*Hilfiker, Mary L., University of Maryland
Hough, Gerard R., Sienna College
*Keller, Brian D., Johns Hopkins University
Laethem, Tom, Oakland University
Laskin, Jeffrey, New York University
*McDermott, Christine E., University of Tennessee
*Morgan, William C., Wake Forest University
Murphy, Dennis J., University of Maryland
*Pace, Robert, University of Massachusetts
Perry, Clayton, Swarthmore College
Ragster, LaVerne, University of Miami
*Robins, Didi, Yale University
Rouse, Anne M., University of Delaware
*Scott, Sherry, Yale University
Seeley, Maggie, University of California, San Diego
*Senseman, David, Princeton University
*Skvirsky, Rachel, Oberlin College
Stuler, Helena, Rice University
Swank, Sarah, University of Southern California
*Winfield, Jeffrey, University of California, Santa Barbara
*Yen, Duen, Johns Hopkins University

NEUROBIOLOGY

Brownell, Philip H., University of California, Riverside
Fishman, Paul S., Yale University
Hall, Dr. Linda, University of British Columbia
Monsell, Edwin M. III, Duke University Medical Center
Olson, Suellen A., Wayne State University
Ross, Dr. William N., Columbia University
Sanchez, Dr. Pompilio H., Del Instituto Politecnico Nacional Mexico
Young, Wise, University of Iowa

FRONTIERS IN RESEARCH AND TEACHING PROGRAM

Alfred, Lawrence, Federal City College
Anekwe, Gregory E., Tuskagee Institute
Clark, Vernon, North Carolina Central University
Cornwell, Catherine, Massachusetts Institute of Technology
Ferguson, Thomas, Delaware State College

MIKULECKY, DONALD C., Meharry Medical College
STEPHENS, LEE B., JR., California State College
TOWNSEL, JAMES G., Harvard Medical School
WALKER, CHARLES A., Tuskegee Institute
WHEATON, GLADYS, Harvard University

3. FELLOWSHIPS AND SCHOLARSHIPS, 1972

The Crocker Scholarship:

JAMES A. ROMESSER, Ecology Course
GAIL L. COTTET, Marine Botany Course

Bio Club Scholarship:

HELENA E. STULER, Invertebrate Zoology

Gary H. Calkins Scholarship:

GERARD R. HOUGH, Invertebrate Zoology

4. TRAINING PROGRAMS

FERTILIZATION AND GAMETE PHYSIOLOGY RESEARCH TRAINING PROGRAM

I. INSTRUCTORS

CHARLES B. METZ, University of Miami, Program Director
JOHN CHAMBERLAIN, University of Washington
GERTRUDE W. HINSCH, University of Miami
MARCO CRIPPA, University of Geneva, Switzerland
ALLEN W. SCHUETZ, Johns Hopkins University
WILLIAM H. TELFER, University of Pennsylvania
ROBIN A. WALLACE, Oak Ridge National Laboratory

II. ASSISTANTS

MRS. ANGELA O'RAND, Program Secretary

III. TRAINEES

AL-HAJJ, HAMEED A., University of Houston
BAST, ROBERT, University of Pennsylvania
BENNETT, DIANE C., University of Minnesota
CLOUD, JOSEPH G., University of Wisconsin
FOX, LINDA, University of Tennessee
GANION, LARRY R., Ball State University
HAGEDORN, HENRY H., University of Connecticut
HOWE, CRAIG, Cambridge University
JOHNSON, CRACELYNN A., Temple University
LEVINSON, JOHN W., University of Michigan
NICKOL, JOANNE, Oak Ridge National Laboratory
PARENT, JAMES BRIAN, University of Virginia
ROSENBERG, PAUL A., Albert Einstein College of Medicine
SEDENSKY, MARGARET, University of Colorado
SELMAN, KAY ELLEN, University of Virginia
SHAY, JERRY W., University of Kansas
SIU, CHI HUNG, University of Chicago
STEINBERG, MARK, University of Pennsylvania

IV. LECTURES

M. BEDFORD	Significance of disulfied cross-links in sperm structure
R. WALLACE	Yolk formation in amphibians
R. E. MANCINI	Immunological factors affecting animal and human spermatogenesis
D. SKINNER	Characterization of crustacean macromolecules synthesized *in vivo* and *in vitro*
W. D. RUSSELL-HUNTER	Adaptive and experimental aspects of sex changes in mollusks
M. TELFER	Biological and social implications of human non-disjunction
I. CALLARD	Hormones and the control of reptilian reproduction
L. NELSON	What motivates a sperm?
J. PIATIGORSKY	Initiation of lens fiber formation *in vivo* and in tissue culture

V. SPECIAL LECTURES

JOHN BISHOP	Reiterated and nonreiterated DNA sequences

EXCITABLE MEMBRANE TRAINING PROGRAM

I. CONSULTANTS

K. S. COLE, National Institute of Neurological Diseases and Stroke, National Institutes of Health
L. J. MULLINS, University of Maryland, School of Medicine
W. J. ADELMAN, JR., National Institute of Neurological Diseases and Stroke, National Institutes of Health

II. INSTRUCTORS AND LECTURERS

D E. GOLDMAN, Program Director, Medical College of Pennsylvania
J. W. MOORE, Program Co-director, Duke University Medical Center
D. L. GILBERT, National Institute of Neurological Diseases and Stroke, National Institutes of Health
L. J. MULLINS, University of Maryland, School of Medicine
W. K. CHANDLER, Yale University School of Medicine
W. J. ADELMAN, JR., National Institute of Neurological Diseases and Stroke, National Institutes of Health
T. NARAHASHI, Duke University Medical Center
G. D. PAPPAS, Albert Einstein College of Medicine
J. K. BLASIE, University of Pennsylvania
E. J. MASORO, Medical College of Pennsylvania
L. E. MOORE, Case Western Reserve University, School of Medicine
I. TASAKI, National Institute of Mental Health
L. B. COHEN, Yale University School of Medicine
J. Y. LETTVIN, Massachusetts Institute of Technology
P. MUELLER, Eastern Pennsylvania Psychiatric Institute
W. R. LOEWENSTEIN, University of Miami Medical School
C. L. PROSSER, University of Illinois
M. L. WOLBARSHT, Duke University Medical Center
F. A. DODGE, IBM Corp.
R. R. LLINAS, University of Iowa
C. M. ARMSTRONG, University of Rochester

III. LABORATORY ASSISTANTS

PETER GREIF, Haverford College
GEORGE STETTEN, Harvard College

IV. TRAINEES

T. B. BEGENESICH, University of Maryland
E. M. CHOY, Case Western Reserve University

J. M. J. Corless, Duke University
J. F. Fohlmeister, University of Minnesota
D. J. Goldberg, Yale University
D. L. McIlwain, University of North Carolina, Chapel Hill
D. C. Mikulecky, Meharry Medical College
J. K. Randolph, Haverford College
R. B. Robinson, University of Illinois
J. G. Rutherford, Dalhousie University
L. A. Stein, University of Pennsylvania

V. Lectures

D. E. Goldman	The physico-chemical background of membrane transport I.
	The physico-chemical background of membrane transport II.
	The physico-chemical background of membrane transport III.
	Some electrical properties of membranes
J. W. Moore	Operational amplifiers
	Electrometers and microelectrodes
	Voltage clamp circuits
D. L. Gilbert	Squid biology and dissection
L. J. Mullins	Passive membrane transport
	Active membrane transport
	Nitella
W. K. Chandler	Currents in the voltage clamp
	The Hodgkin-Huxley formulation
	Univalent ions and selectivity
J. W. Moore	TTX as an analytical tool
D. L. Gilbert	Calcium ions and surface charges
D. E. Goldman	The action of polyvalent cations
W. J. Adelman	The role of the periaxonal space
T. Narahashi	The action of drugs and anesthetics
	The action of toxins as lytic enzymes
G. D. Pappas	Electron microscopy of membranes
J. K. Blasie	X-ray diffraction and excitable membranes
W. J. Masoro	The chemical composition of membranes
D. E. Goldman	Non-electrical stimulation of nerve
L. E. Moore	Temperature pump studies in the voltage clamp
I. Tasaki	Fluorescent labelling of squid axons
L. B. Cohen	Optical studies of axons
F. A. Dodge	Cable theory
L. E. Moore	Myelinated nerve
	Muscle
J. Y. Lettvin	A transistor model for nerve membranes
P. Mueller	Artificial membranes
D. E. Goldman	Excitability models I.
	Excitability models II.
	Excitability models III.
F. A. Dodge	Electrical noise in membranes
C. M. Armstrong	Models for the g_k process
W. K. Chandler	Models for the g_{Na} process
C. L. Prosser	Non-striated muscle
M. L. Wolbarsht	Receptors and transducers
	The goldfish eye
F. A. Dodge	*Limulus* eye I
	Limulus eye II

5. TABULAR VIEW OF ATTENDANCE, 1968–1972

	1968	*1969*	*1970*	*1971*	*1972*
INVESTIGATORS—TOTAL	528	566	532	554	561
Independent	281	310	324	322	328
Library Reader	76	68	73	76	76
Research Assistants	171	188	135	156	157
STUDENTS—TOTAL	122	118	142	130	119
Invertebrate Zoology	39	35	41	29	38
Embryology	20	20	28	28	19
Physiology	30	30	31	33	31
Experimental Botany	15	16	19	22	14
Ecology	18	17	23	18	17
TRAINEES—TOTAL	17	29	33	44	46
TOTAL ATTENDANCE	667	713	707	728	726
Less Persons represented in two categories	7	5	0	0	1
	660	708	707	728	725
INSTITUTIONS REPRESENTED—TOTAL	169	187	191	219	210
FOREIGN INSTITUTIONS REPRESENTED	23	24	21	27	25

6. INSTITUTIONS REPRESENTED, 1972

Agnes Scott College
Albert Einstein College of Medicine
American Museum of Natural History
Amherst College
Argonne National Laboratory
Ball State University
Boston City Hospital
Boston University
Boston University School of Medicine
Brandeis University
Brooklyn College, The City University of New York
Brown University
California, University of, Berkeley
California, University of, Davis
California, University of, Irvine
California, University of, Los Angeles
California, University of, Riverside
California, University of, San Diego
California, University of, Santa Barbara
California, University of, Santa Cruz
California Institute of Technology
California State College
Carleton College
Carnegie-Mellon University
Case Western Reserve University
Chicago, University of
Cincinnati, University of
City College, The City University of New York
Clark University
College of William & Mary
Colorado, University of
Colorado, University of, Medical Center
Columbia University
Columbia University, College of Physicans and Surgeons
Connecticut, University of
Connecticut, University of, Health Center
Connecticut, University of, Medical School
Connecticut College
Cornell University Medical College
Dartmouth College
Delaware, University of
Delaware State College
De Paul University
De Pauw University
Drew University
Duke University
Duke University Medical Center
Earlham College
Emory University
Fairleigh Dickinson University
Federal City College
Florida, University of
Florida Atlantic University
Florida State University
Goddard College
Goucher College
Georgia, University of
Harvard College
Harvard Medical School
Harvard University
Hawaii, University of

Herbert Lehman College, The City University of New York
Houston, University of
Howard University
Hunter College, The City University of New York
IBM Research
Illinois, University of
Illinois Institute of Technology
Indiana University
Institute for Cancer Research, The
Institute for Muscle Research
Iowa, University of
Ithaca College
Jersey City State College
Johns Hopkins University, The
Johns Hopkins University, The, School of Medicine
Kansas, University of
Kansas State University
Kentucky, University of
Keuka College
Louisiana State University
Lowell Technological Institute
Marine Research Foundation, Inc.
Maryland, University of
Maryland, University of, School of Medicine
Massachusetts, University of
Massachusetts, University of, at Boston
Massachusetts General Hospital
Massachusetts Institute of Technology
Medical College of Ohio at Toledo
Medical College of Pennsylvania
Meharry Medical College
Miami, University of
Michigan, University of
Michigan State University
Millersville State College
Minnesota, University of
Missouri, University of
Mt. Sinai School of Medicine, The City University of New York
National Academy of Science
National Institute of Mental Health
National Institutes of Health
New Hampshire, University of
New York Blood Center, The
New York Medical College
New York State Psychiatric Institute
New York University
New York University Medical College
North Carolina, University of
North Carolina Central University
North Carolina State University, Raleigh
Northwestern University
Notre Dame, University of
Oak Ridge National Laboratory
Oakland University
Oberlin College
Ohio State University
Ohio University
Oregon, University of
Oregon State University
Pennsylvania, University of
Pennsylvania, University of, School of Medicine
Pennsylvania State University
Pittsburgh, University of
Pomona College
Princeton University
Public Health Research Institute of City of New York Inc.
Purdue University
Queens College, The City University of New York
Quincy College
Ramapo College of New Jersey
Reed College
Rensselaer Polytechnic Institute
Rice University
Rochester, University of
Rochester, University of, Medical School
Rockefeller University, The
Rutgers—The State University
St. Louis University
Siena College
Southern Methodist University
Spelman College
Stanford University
State University of New York, Downstate Medical Center
State University of New York, Upstate Medical Center
State University of New York at Albany
State University of New York at Buffalo
State University of New York at Stony Brook
Swarthmore College
Syracuse University
T. J. Watson Research Center
Temple University
Tennessee, University of
Tennessee, University of, Medical Units
Texas, University of
Texas, University of, Austin
Texas, University of, Houston
Texas Christian University
Trinity College
Tufts University
Tulane University
Tuskegee Institute
Union College
Union University, Albany Medical College of
Upsala College
Vanderbilt University School of Medicine
Vassar College
Veterans Administration Hospital, Baltimore, Maryland
Veterans Administration Hospital, Brooklyn

Virginia, University of
Virginia State College
Wake Forest University
Washington, University of
Washington University
Washington University School of Medicine
Wayne State University
Wesleyan University
Western State College
Wilson College
Wisconsin, University of
Wistar Institute
Woods Hole Oceanographic Institution
Yale University
Yale University School of Medicine

Foreign Institutions Represented, 1972

Barcelona, University of, Spain
British Columbia, University of, Canada
Cambridge, University of, England
Caribbean Marine Biological Institute, Netherlands
Chile, University of, Chile
CNR Institute of Marine Biology, Italy
Colegio del Sagrado Corazon, Uruguay
Del Instituto Politecnico Nacional, Mexico
Geneva, University of, Switzerland
Hebrew University of Jerusalem, Israel
Institute of Molecular Biology, France
Lausanne, University of, Switzerland
Leeds, University of, England
Medical Research Council, England
Montreal, University of, Canada
Ottawa, University of, Canada
Padua, University of, Italy
Puerto Rico, University of, at Rio Piedras, Puerto Rico
Tel-Aviv University, Israel
Toronto, University of, Canada
Universidad Central de Venezuela, Venezuela
Universita degli Studi di Milano, Italy
Universite Laval, Canada
University College, England
Zoological Laboratory, Utrecht, Netherlands

7. Friday Evening Lectures, 1972

June 28

Thomas Eisner.................. Better living through chemistry—insect style
Cornell University

July 6

Douglas R. Wilkie............. Muscle as a chemical engine—part I
University College, London;
Alexander Forbes Lecturer at MBL

July 7

Douglas R. Wilkie............. Muscle as a chemical engine—part II

July 14

Max Burger.................... Tumor cell membranes: with beans in search of the culprit
Princeton University

July 21

Philip B. Dunham.............. Na-K pump sites: control of their numbers by genes and antibodies
Syracuse University

July 28

William H. Telfer............. The intercellular bridges of animal germ cells
University of Pennsylvania

August 4

Lynn Margulis................. Symbiosis and cell evolution
Boston University

August 11

Timothy Goldsmith Yale University	Compound eyes and the visual world of arthropods

August 18

Harlyn Halvorson Brandeis University	Control of meiosis in yeast

8. Members of the Corporation, 1972

Including Action of 1972 Annual Meeting

Life Members

Adolph, Dr. Edward F., University of Rochester School of Medicine and Dentistry, Rochester, New York 14627
Behre, Dr. Elinor M., Black Mountain, North Carolina 28711
Bertholf, Dr. Lloyd M., 1228 Gettysburg Drive, Bloomington, Illinois 61701
Bodansky, Dr. Oscar, Department of Biochemistry, Memorial Cancer Center, 444 East 68 Street, New York, New York 10021
Bradley, Dr. Harold C., 2639 Durant Avenue, Berkeley, California 94704
Brody, Donald, 522 Fifth Avenue, New York, New York 10018
Burdick, Dr. C. Lalor, The Lalor Foundation, 4400 Lancaster Pike, Wilmington, Delaware 19805
Claff, Dr. C. Lloyd, 506 N. Warren, Brockton, Massachusetts 02403
Cole, Dr. Elbert C., 2 Chipman Park, Middlebury, Vermont 05753
Cowdry, Dr. E. V., 4580 Scott Avenue, St. Louis, Missouri 63110
Dawson, Dr. A. B., 12 Scott Street, Cambridge, Massachusetts 02138
Diller, Dr. Irene C., 2417 Fairhill Avenue, Glenside, Pennsylvania 19038
Diller, Dr. William F., 2417 Fairhill Avenue, Glenside, Pennsylvania 19038
Hamburger, Dr. Viktor, Department of Zoology, Washington University, St. Louis, Missouri 63110
Hess, Dr. Walter, 787 Maple Street, Spartanburg, South Carolina 29302
Hibbard, Dr. Hope, 366 Reamer Place, Oberlin, Ohio 44074
Hisaw, Dr. F. L., 5925 S. W. Plymouth Drive, Corvallis, Oregon 97330
Hollaender, Dr. Alexander, Biology Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee 37830
Irving, Dr. Laurence, University of Alaska, College, Alaska 99701
Lowther, Dr. Florence, Barnard College, New York, New York 10027
MacDougall, Dr. Mary Stuart, Mt. Vernon Apartments, 423 Clairmont Avenue, Decatur, Georgia 30030
Malone, Dr. E. F., 6610 North 11th Street, Philadelphia, Pennsylvania 19126
Manwell, Dr. Reginald D., Department of Biology, Syracuse University, Syracuse, New York 13210
Page, Dr. I. H., Cleveland Clinic, Euclid at E. 93rd Street, Cleveland, Ohio 44106
Payne, Dr. Fernandus, Indiana University, Bloomington, Indiana 47405
Plough, Dr. H. H., 15 Middle Street, Rt. 1, Amherst, Massachusetts 01002
Pollister, Dr. A. W., Department of Zoology, Columbia University, New York, New York 10027

Pond, Samuel E., 53 Alexander Street, Manchester, Connecticut 06044
Porter, Dr. H. C., University of Pennsylvania, Philadelphia, Pennsylvania 19104
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V. REPORT OF THE LIBRARIAN

Another gift from the MBL Associates of approximately $6,000 was given to the library at the end of the 1972 summer season. To date the library has purchased 452 books with the Associates' 1971 gift. This gift is making a definite contribution to the book section. Our ecology section is certainly much improved.

The sale of our catalog, published by the G. K. Hall Company has increased the number of the inter-library loan requests received in 1972. We processed 6,714 requests from the following: 260 university and college libraries, 67 government agencies, 90 industrial firms, 13 marine laboratories and 60 institutions in 16 foreign countries.

During the summer an Inter-Institutional Library Committee was appointed consisting of three scientists each from both the MBL and the Woods Hole Oceanographic Institution. The latter is expanding its facilities to a campus at Quissett and there has been concern about the availability of the library to the scientists at WHOI. It was determined by all institution representatives that the library should not be divided with part of the collection housed at Quissett. The Oceanographic will continue to use the MBL Library as the repository for their collection and methods for rapid service to WHOI scientists are being studied.

The library is currently receiving 2,529 serial titles and holdings now total 148,974 volumes.

VI. REPORT OF THE TREASURER

The market value of the General Endowment Fund and the Library Fund at December 31, 1972, amounted to $2,579,583, and the corresponding securities are entered in the books at a value of $1,624,597. This compares with values of $2,294,949 and $1,555,338, respectively, at the end of the preceding year. The average yield on the securities was 3.64% of the market value and 5.78% of the book value. Uninvested principal cash was $1850. Classification of the securities held in the Endowment Fund appears in the Auditor's Summary of Investments.

The market value of the Pooled Securities at December 31, 1972, amounted to $1,238,943 as compared to book values of $922,227. These figures compare with values of $870,538 and $667,280, respectively, at the close of the preceding year. The average yield on the securities was 3.12% of the market value and 4.20% of the book value. Uninvested principal cash was in the amount of $30,986.

The proportionate interest in the Pool Fund Account of the various funds, as of December 31, 1972, is as follows:

Pension Funds	28.32%
General Laboratory Investment	28.88%
F. R. Lillie Memorial Fund	2.10%
Other:	
Bio Club Scholarship Fund	.55%
Rev. Arsenius Boyer Scholarship Fund	.66%
Gary N. Calkins Fund	.63%
Allen R. Memhard Fund	.12%
Lucretia Crocker Fund	2.28%
E. G. Conklin Fund	.38%
Jewett Memorial Fund	.20%
M. H. Jacobs Scholarship Fund	.27%
Herbert W. Rand Fellowship	19.41%
Mellon Foundation	9.16%
Mary Rogick Fund	2.00%
Swope Foundation	5.04%

Donations from MBL Associates for 1972 amounted to $10,755 as compared with $10,507 for 1971. Unrestricted gifts from foundations, societies and companies amounted to $56,546.

During the year we administered the following grants and contracts:

Investigators	*Training*	*MBL Institutional*
3 NIH	3 NIH	2 NSF
4 NSF	1 Research Corp	
1 EPA	1 Sloan	
1 MSPC		
1 NOAA		
10	5	2

Most of the federally funded grants and contracts provided for reimbursement of indirect costs on a cost per square foot basis for the laboratory space assigned to each research project. A rate of $10.00 per square foot was in effect until October, when it was superseded for new grants by a provisional rate of $12.25 per square foot. Training courses supported by NIH grants were funded for indirect costs as a rate of 8% of allowable direct costs.

The following is a statement of the auditors:

To the Trustees of Marine Biological Laboratory, Woods Hole, Massachusetts:

We have examined the balance sheet of Marine Biological Laboratory as of December 31, 1972 and the related statements of operating expenditures and income and funds for the year then ended. Our examination was made in accordance with generally accepted auditing standards, and accordingly included such tests of the accounting records and such other auditing procedures as we considered necessary in the circumstances. We previously examined and reported on the 1971 financial statements.

In our opinion, the aforementioned financial statements (pages 77 to 81) present fairly the financial position of Marine Biological Laboratory at December 31, 1972 and 1971, and its operating expenditures and income for the years then ended, and the changes in its funds for the year ended December 31, 1972, in conformity with the accounting principles referred to in Note A to the financial statements applied on a consistent basis.

The supplementary schedules (page 82) included in this report were obtained from the Laboratory's records in the course of our examination and, in our opinion, are fairly stated in all material respects in relation to the financial statements taken as a whole.

Boston, Massachusetts
March 23, 1973

Lybrand, Ross Bros. and Montgomery

MARINE BIOLOGICAL LABORATORY

BALANCE SHEET December 31, 1972 and 1971

Investments	*1972*	*1971*
Investments held by Trustee:		
Securities, at cost (approximate market quotation, 1972—$2,579,583; 1971—$2,294,949)	$ 1,624,597	$ 1,555,338
Cash	1,850	7,757
	1,626,447	1,563,095
Investments of other endowment and unrestricted funds:		
Pooled investments, at cost (approximate market quotation, 1972—$1,238,943; 1971—$870,538), less $5,728 temporary investment of current fund cash in 1971	922,227	661,552
Other investments	1,094,752	725,151
Cash	31,516	2,295
Due from current fund	131,927	94,412
	$ 3,806,869	$ 3,046,505
Plant Assets		
Land, buildings, library and equipment, at cost	12,453,416	12,443,510
Less allowance for depreciation (Note A)	2,444,052	2,161,247
	10,009,364	10,282,263
Investments, at cost (approximate market quotation in 1971—$537,115)	—	737,881
	$10,009,364	$11,020,144
Current Fund Assets		
Cash	388,130	199,877
Temporary investment in pooled securities	—	5,728
Accounts receivable (U. S. Government, 1972—$88,186; 1971—$69,395)	235,903	248,396
Inventories of supplies and bulletins	43,514	44,999
Other assets	5,581	7,798
Due to endowment funds	(131,927)	(94,412)
	$ 541,201	$ 412,386

MARINE BIOLOGICAL LABORATORY

Balance Sheet December 31, 1972 and 1971

Invested Funds

	1972	*1971*
Endowment funds given in trust for benefit of Marine Biological Laboratory	$ 1,626,447	$ 1,563,095
Endowment funds for awards and scholarships:		
Principal	427,702	427,702
Unexpended income	60,795	55,591
	488,497	483,293
Unrestricted funds functioning as endowment	1,435,918	779,190
Retirement fund	316,512	279,407
Pooled investments—accumulated loss	(60,505)	(58,480)
	$ 3,806,869	$ 3,046,505

Plant Funds

Funds expended for plant, less retirements	12,453,416	12,443,510
Less allowance for depreciation charged thereto	2,444,052	2,161,247
	10,009,364	10,282,263
Unexpended plant funds	—	737,881
	$10,009,364	$11,020,144

Current Fund Liabilities and Balances

Accounts payable and accrued expenses	26,333	31,485
Advance subscriptions	28,293	34,338
Unexpended grants—research	77,774	47,837
Unexpended balances of gifts for designated purposes	87,949	39,725
Current fund	320,852	259,001
	$ 541,201	$ 412,386

The accompanying note is an integral part of the financial statements.

Note A.—*Accounting Principles*: The following accounting principles have been reflected in the accompanying financial statements:

1. Investments are stated at cost.
2. Investment income is recorded on a cash basis.
3. Operating income is recorded when earned.
4. Expenses are recorded on an accrual basis.
5. Depreciation has been provided for plant assets at annual rates ranging from 1% to 5% of the original cost of the assets.

MARINE BIOLOGICAL LABORATORY

STATEMENT OF OPERATING EXPENDITURES AND INCOME

Years ended December 31, 1972 and 1971

	1972						
	Salaries and Wages	*Other Costs and Expenses*	*Depreciation (Note A)*	*Total*	*Charged to Grants*	*1972 Total*	*1971*
Operating expenditures:							
Instruction		$ 28,482	$ 75,584	$ 104,066	$271,567	$ 375,633	$ 418,315
Research		43,067	81,786	124,853	311,388	436,241	459,637
Dormitories	$ 21,151	44,745	100,980	166,876		166,876	145,428
Dining		108,745		108,745		108,745	89,637
Library	41,240	15,312	17,701	74,253		74,253	68,280
Back sets, serials and binding		60,674		60,674		60,674	49,707
Biological Bulletin	5,677	49,730		55,407		55,407	49,342
Support services:							
Apparatus	63,446	57,515		120,961		120,961	114,091
Supply	84,989	65,533	4,407	154,929		154,929	145,079
Administration	100,249	96,843		197,092		197,092	183,008
Plant operation	163,781	145,582	2,347	311,710		311,710	277,610
Grant expenditures for support services					19,296	19,296	8,130
Other		38,293		38,293		38,293	34,800
	$480,533	$754,521	$282,805	1,517,859	602,251	2,120,110	2,043,064

MARINE BIOLOGICAL LABORATORY

Statement of Operating Expenditures and Income

Years ended December 31, 1972 and 1971

	Fees	Other				
Income:						
Instruction	$ 87,520		87,520	271,567	359,087	400,996
Research	231,643		231,643	311,388	543,031	549,693
Dormitories		$179,321	179,321		179,321	146,559
Dining		121,083	121,083		121,083	101,419
Library	26,952	39,407	66,359		66,359	58,639
Biological Bulletin		57,139	57,139		57,139	56,852
Support services:						
Apparatus		49,724	49,724		49,724	45,847
Supply		62,973	62,973		62,973	68,058
Administration		21,554	21,554		21,554	19,103
Investment income		150,864	150,864		150,864	131,615
Gifts used for current expense		67,302	67,302		67,302	52,532
Allowance for indirect costs		39,393	39,393		39,393	63,362
Grants for support services				19,296	19,296	8,130
Other		6,937	6,937		6,937	1,670
	$346,115	$795,697	1,141,812	$602,251	1,744,063	1,704,475
Excess of current expenditures and depreciation over current income			376,047		376,047	338,589
Reduction in plant funds for depreciation			282,805		282,805	264,533
Excess current expenditures			$ 93,242		$ 93,242	$ 74,056

The accompanying note is an integral part of the financial statements.

MARINE BIOLOGICAL LABORATORY

STATEMENT OF FUNDS

Year ended December 31, 1972

	Balance December 31, 1971	*Gifts and Other Receipts*	*Invest-ment Income*	*Used for Current Expenses*	*Transfers*		*Other Ex-pend-itures*	*Balance December 31, 1972*
					To	*From*		
Invested funds	$3,046,505	$114,257	$175,508	$150,864	$ 5,728 816,000	$165,000	$35,265	$3,806,869
Unexpended plant funds	$ 737,881	78,119				816,000		—
Unexpended research grants	$ 47,837	670,999		641,062				$ 77,774
Unexpended gifts for designated purposes	$ 39,725	65,333		10,755		5,728	626	$ 87,949
Current fund	$ 259,001	(93,242)(1)			165,000		9,907	$ 320,852
		$835,466	$175,508	$802,681	$986,728	$986,728	$45,798	
Plant gifts		78,119						
Other gifts and receipts		65,333						
Grants for research, training and support		670,999						
Appropriated from current income and other		114,257						
(1) Excess of current expenditures over income		(93,242)						
		$835,466						
Appropriation for dormitory—dining hall							9,907	
Scholarship awards							9,875	
Payments to pensioners							10,393	
Loss on sale of securities							14,997	
Other							626	
							$45,798	

The accompanying note is an integral part of the financial statements.

MARINE BIOLOGICAL LABORATORY

Summary of Investments

December 31, 1972

	Cost	*Per-cent of Total*	*Market Quotations*	*Per-cent of Total*	*Investment Income 1972*
Securities held by Trustee:					
General endowment fund:					
U. S. Government securities	$ 25,065	1.9	$ 25,500	1.2	$ 1,813
Corporate bonds	640,163	48.2	570,493	27.0	31,106
Preferred stocks	74,643	5.6	46,400	2.2	3,096
Common stocks	587,528	44.3	1,470,676	69.6	42,191
	1,327,399	100.0	2,113,069	100.0	78,206
General educational board endowment fund:					
U. S. Government securities	51,112	17.2	52,020	11.2	3,698
Other bonds	145,965	49.1	117,485	25.2	6,710
Preferred stocks					145
Common stocks	100,121	33.7	297,009	63.6	5,066
	297,198	100.0	466,514	100.0	15,619
Total securities held by Trustee	$1,624,597		$2,579,583		93,825
Investments of other endowment and unrestricted funds:					
Pooled investments:					
U. S. Government securities	68,247	7.4	68,517	5.5	5,493
Corporate bonds	274,200	29.7	233,984	18.9	10,969
Preferred stocks	26,482	2.9	22,500	1.8	3,004
Common stocks	553,298	60.0	913,942	73.8	19,238
	922,227	100.0	$1,238,943	100.0	38,704
Other investments:					
U. S. Government securities	27,893				1,133
Other bonds	15,029				750
Common stocks	581,410				20,887
Preferred stocks	2,871				104
Real estate	17,549				—
Short-term commercial notes	450,000				27,675
	1,094,752				50,549
Total investments of other endowment and unrestricted funds	$2,016,979				
Total					183,078
Custodian's fees charged thereto					(7,570)
Total investment income					$175,508

Reference: *Biol. Bull.*, **145**: 83–90. (August 1973)

GENE-ENZYME VARIATION IN THREE SYMPATRIC SPECIES OF *LITTORINA*

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The three littorinid species of the northwestern Atlantic occupy distinct but overlapping habitats in the rocky intertidal zone (Haseman, 1911), from Labrador to the Virginias. The most ubiquitous of these species, *Littorina littorea*, inhabits the middle intertidal, and is believed to be a recent immigrant from western Europe (Sumner, Osborn, and Cole, 1911). This species deposits small egg cases directly into the sea, where development, taking several weeks, proceeds up to the veliger stage. Free swimming larvae then complete their embryonic development in the water column (Purchon, 1968). After some time juvenile adults settle onshore and assume the relatively sedentary life of adult snails. *Littorina obtusata* inhabits the lower intertidal zone subsisting almost entirely off the microflora adhering to either of two algal genera, *Fucus* and *Ascophyllum* (Haseman, 1911). Large gelatinous egg cases of *L. obtusata* are cemented onto the anchored algae, where development proceeds through the larval stages, and the emerging form is a crawling juvenile adult (Purchon, 1968). The third common species, *Littorina saxatilis*, inhabits the upper intertidal and supratidal zones, grazing upon the microflora of encrusted rocks (Haseman, 1911). In contrast to its two sympatric species, *L. saxatilis* has evolved oviviviparity. Eggs are retained within the females' body cavity where development proceeds to the juvenile adult stage (Purchon, 1968).

One possible consequence of these divergent reproductive patterns is immediately apparent. The oviviviparity of *L. saxatilis,* along with its preference for the high intertidal, must severely restrict larval dispersal, and hence limit the potential for interpopulation gene flow. A similar situation may hold for *L. obtusata,* whose eggs and adults face only limited dispersal resulting from the infrequent dislodgement of anchored algae. In contrast, the extensive pelagic period of *L. littorea* eggs and larvae must permit extensive dispersal along the coastal range, in the direction of the predominant longshore ocean currents.

In this study an attempt was made to evaluate the actual consequences of these divergent dispersal capabilities on the distribution of genetic variability in these species, and among their individual populations. The technique of gel electrophoresis was employed, and results from three esterase polymorphisms are discussed.

MATERIALS AND METHODS

Collection sites—and sampling

Fifteen rocky shore localities were sampled, covering a range of about 500 air miles. The localities (Fig. 1) include Charlottetown, Prince Edward Island

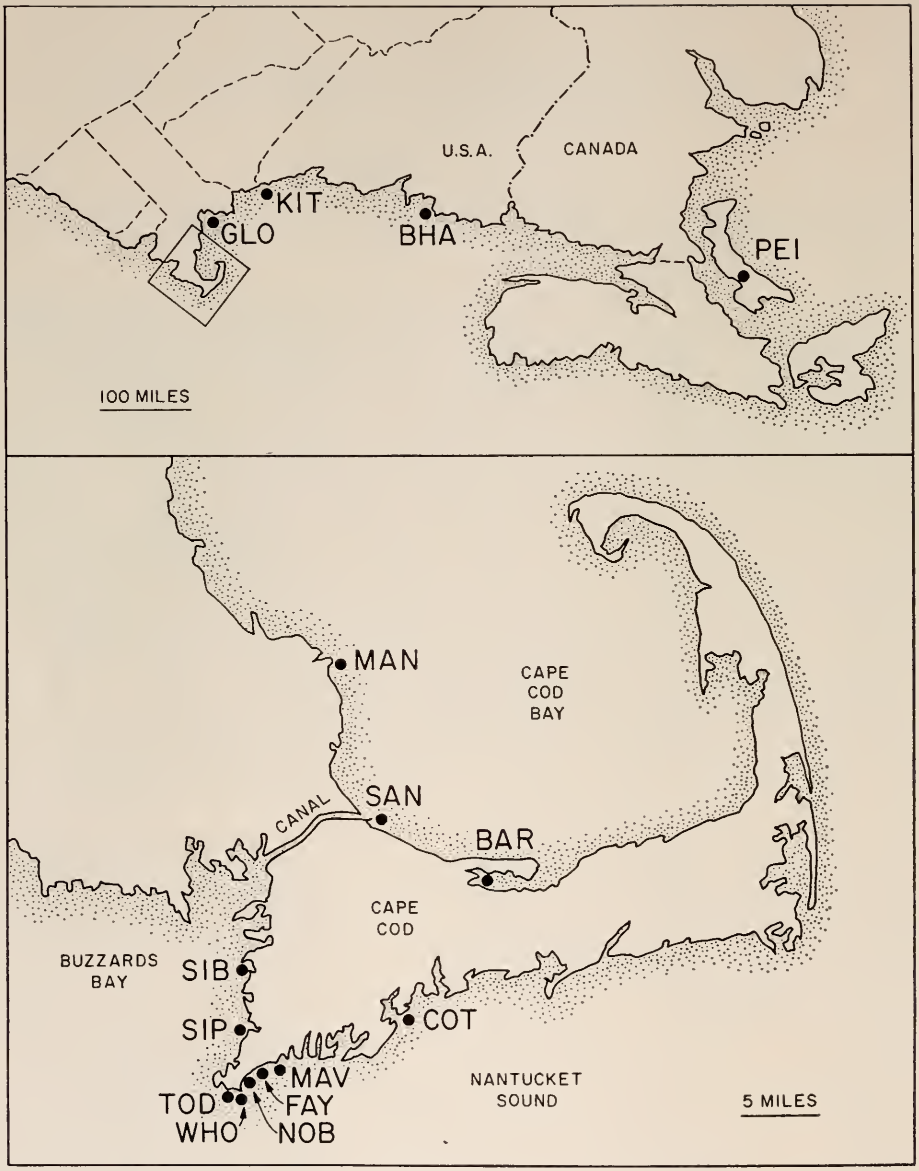
U.S.A.
CANADA
KIT
GLO
BHA
PEI
100 MILES
MAN
CAPE
COD
BAY
CANAL
SAN
BAR
CAPE
COD
BUZZARDS
BAY
SIB
SIP
COT
MAV
TOD
FAY
WHO
NOB
NANTUCKET
SOUND
5 MILES

FIGURE 1. Map designations of the 15 collection sites at which *Littorina* populations were sampled. Site designations are explained in Methods.

(PEI); Bar Harbor, Maine (BHA); Kittery, Maine (KIT); Gloucester, Massachusetts (GLO); Manomet Point, Massachusetts (MAN); Sandwich, Massachusetts (SAN); Barnstable, Massachusetts (BAR); Silver Beach, Massachusetts (SIB); Sippewissett, Massachusetts (SIP); the municipal dock (TOD), and fisheries beach (WHO) in Woods Hole, Massachusetts; Nobska Point

(NOB), Fay Beach (FAY), and Mara Vista (MAR) in Falmouth, Massachusetts; and Cotuit, Massachusetts (COT).

L. littorea was collected from 13 of the 15 sites (excluding MAV and FAY); *L. obtustata* from 14 sites (excluding SIB) and *L. saxatilis* from 12 sites (excluding WHO, MAV and COT). Individuals were maintained alive in running sea water until electrophoretic analysis was performed. Between 6 and 43 individuals of each species, from each population, were analyzed electrophoretically for each enzyme system.

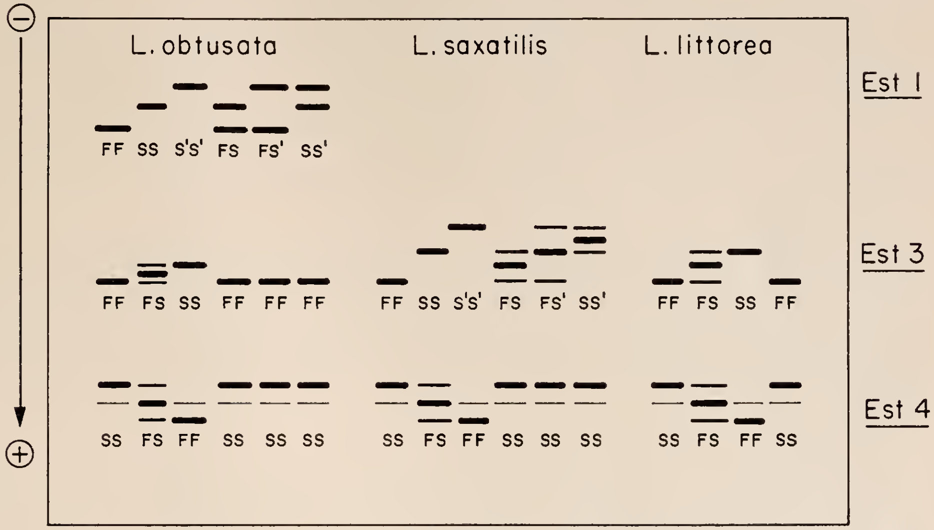

FIGURE 2. Electrophoretic patterns of homozygous and heterozygous genotypes at the three polymorphic esterase loci in the three species of *Littorina*. Enzyme migration in all cases was toward the anode (bottom).

Electrophoresis

Individual snails were removed from their shells and homogenized in two volumes of cold 10% sucrose solution. Homogenates were centrifuged 6000 × *g* for 10 minutes and aliquots of the supernatant applied directly onto the gels. In many cases the supernatants were stored frozen (−18° C) before analysis. Procedures for acrylamide gel electrophoresis and esterase staining followed the method described by Hubby and Lewontin (1966). Internal standardizations of mobility variants were made for each new population sampled, by including TOD individuals of known genotype.

RESULTS

The esterase polymorphisms

Littorina littorea. Four major zones of esterase activity were observed in zymograms of individual *L. littorea*. Est 1 and Est 2 were completely monomorphic for the same allele in all the populations studied. Est 3 and Est 4, shown in

Figure 2, were both polymorphic, each locus having two alleles in the range of populations sampled. Homozygotes at either locus were characterized by a single major band, and heterozygotes displayed a three banded pattern characteristic of a dimeric enzyme in which subunit polypeptides associate at random.

Littorina obtusata. Three major zones of esterase activity were observed from single *L. obtusata* (Fig. 2). At the Est 1 locus three variant alleles were observed among the populations sampled, while the Est 3 and Est 4 polymorphisms consisted of two alleles each. Est 1 heterozygotes displayed two banded patterns, while heterozygotes at the Est 3 and Est 4 loci had the more typical three banded pattern. In all cases homozygotes yielded a single band.

Littorina saxatilis. A variable number of esterase zones were detected in individual homogenates of *L. saxatilis.* Because of the complex banding pattern in the cathodal region of the gel only the two anionic systems were evaluated. At the Est 3 locus in this species three alleles were found at high frequency, and at the Est 4 locus two common alleles were detected. Both gene-enzyme systems showed, in the homozygote, a single band; and in the heterozygote a three band pattern (Fig. 2).

In our buffer system the F allele at the Est 3 locus, and both the F and S alleles at the Est 4 locus, appeared to have indistinguishable mobilities in all three species. Similarly, the S allele at the Est 3 locus had identical mobilities in both *L. littorea* and *L. saxatilis.* The Est 3^S allele of *L. obtusata,* and the Est $3^{S'}$ *L. saxatilis* appeared species specific. These mobility identities, however, do not constitute final proof of their chemical identity.

Allele frequencies in natural populations

Littorina littorea. The distribution of allele frequencies in the 13 populations sampled is presented in Tables I and II. The predominant allele at the Est 3 locus is the F variant. The Est 3^S allele while common (present in 6 of the 13 populations) is maintained at low frequency (0.03 to 0.36) in those populations surveyed. At the Est 4 locus the S allele is fixed in 11 of 13 populations, and nearly so in two others (WHO and BAR).

Littorina obtusata. Allele frequency determinations for the 14 populations of *L. obtusata* sampled are included in Tables I and II. At the Est 1 locus the F allele was found at relatively high frequency in the southern Cape Cod region 0.36 to 0.60), while in populations north of the Cape Cod Canal this allele occurred at lower frequencies (0.02 to 0.40). The most northern population, from PEI, was completely lacking this variant. The Est 1^S allele was observed in all the populations at frequencies ranging from 0.35 to 0.90. The PEI population was characterized by a unique allele, Est $1^{S'}$, whose frequency was 0.57.

At the Est 3 locus both alleles were found at high frequency in all but the FAY population, where Est 3^S was fixed. No pattern of clinal variation could be discriminated. The Est 4 polymorphism was observed in 11 of the populations. In the northern species range the frequency of Est 4^F was low, but in several populations clustered around WHO the Est 4^F allele reached high values (0.44–0.52).

Littorina saxatilis. As was the case for *L. obtusata,* one of the polymorphic loci in *L. saxatilis* showed marked regional differentiation. In the southern Cape

TABLE I

Esterase phenotypes for Littorina littorea, L. obtusata, *and* L. saxatilis *for the 15 localities sampled. Dashed lines indicate the absence of a species at that site. O* refers to other genotypes; in addition, for* L. obtusata, *at PEI. Est 1 are: S′S′ = 9; SS′ = 13.*

Site	*L. obtusata*										*L. saxatilis*									*L. littorea*					
	Est 1				Est 3			Est 4			Est 3						Est 4			Est 3			Est 4		
	FF	FS	SS	O*	FF	FS	SS	FF	FS	SS	FF	FS	SS	FS′	SS′	S′S′	FF	FS	SS	FF	FS	SS	FF	FS	SS
COT	2	16	2	0	0	7	12	4	9	7	—	—	—	—	—	—	—	—	—	13	1	1	0	0	25
MAV	1	8	5	0	2	7	5	0	0	14	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
FAY	2	7	0	1	0	0	10	0	1	9	6	6	9	0	0	0	0	1	23	—	—	—	—	—	—
NOB	5	12	4	0	3	5	13	3	6	11	8	10	6	0	0	0	0	1	23	14	0	0	0	0	20
WHO	5	12	6	1	2	12	8	6	12	15	—	—	—	—	—	—	—	—	—	7	0	0	1	1	35
TOD	2	17	5	0	1	11	12	5	12	6	2	22	5	0	0	0	0	2	20	32	6	0	0	0	43
SIP	8	10	6	0	5	12	9	3	15	6	3	5	5	0	0	0	0	0	13	12	1	1	0	0	15
SIB	—	—	—	—	—	—	—	—	—	—	2	2	3	0	0	0	0	0	7	7	3	3	0	0	26
BAR	4	10	9	0	1	7	16	1	9	14	1	3	4	0	0	0	0	0	8	6	0	0	0	2	12
SAN	2	5	17	0	2	13	9	0	8	16	0	7	8	1	7	0	5	5	14	9	3	0	0	0	12
MAN	0	10	13	0	4	11	9	0	4	20	0	8	6	1	6	3	0	1	23	7	0	0	0	0	9
GLO	0	1	23	0	6	9	9	2	14	8	0	3	4	1	7	8	0	0	24	12	0	0	0	0	12
KIT	0	9	15	0	3	10	11	0	1	23	0	1	4	0	7	1	0	0	15	6	0	0	0	0	6
BHA	2	15	9	0	3	9	12	0	0	24	0	5	8	0	6	4	1	4	17	10	0	0	0	0	17
PEI+	0	0	4	1	1	6	21	0	0	27	6	9	2	2	4	1	0	1	23	14	3	0	0	0	22

LIBRARY MASS.

Cod populations both Est 3^{F} and Est 3^{S} were found at high, and roughly similar frequencies (Tables I and II). Proceeding north from Cape Cod the frequency of the Est 3^{F} allele gradually decreased reaching a minimum value of 0.10 at GLO and KIT and BHA. This reduction was not accompanied by a coordinate increase in Est 3^{S} frequency, but rather by the appearance and gradual increase in frequency of a new variant, Est $3^{S'}$. At PEI the frequency of Est 3^{F} is again high, 0.51, and the frequency of Est $3^{S'}$ low, 0.15. The Est 4 polymorphism was observed in 7 of the 12 populations sampled. The Est 4^{F} allele is generally at low frequency, in all populations, although at the SAN locale a value of 0.31 was recorded.

TABLE II

Esterase allele frequencies for Littorina littorea, L. obtusata *and* L. saxatilis *in the 15 localities sampled. Dashed lines indicate the absence of a species at that site.*
O refers to other rare variants.*

Site	*L. obtusata*								*L. saxatilis*					*L. littorea*			
	Est 1				Est 3		Est 4		Est 3			Est 4		Est 3		Est 4	
	F	S	S′	0*	F	S	F	S	F	S	S′	F	S	F	S	F	S
COT	0.50	0.50	0	0	0.18	0.72	0.43	0.57	—	—	—	—	—	0.97	0.03	0	1.0
MAV	0.36	0.64	0	0	0.39	0.61	0	1.0	—	—	—	—	—	—	—	—	—
FAY	0.60	0.35	0	0.05	0	1.0	0.05	0.95	0.40	0.60	0	0.02	0.98	—	—	—	—
NOB	0.52	0.48	0	0	0.26	0.74	0.30	0.70	0.54	0.46	0	0.03	0.97	1.0	0	0	1.0
WHO	0.50	0.49	0	0.01	0.35	0.65	0.52	0.48	—	—	—	—	—	1.0	0	0.03	0.97
TOD	0.44	0.56	0	0	0.27	0.73	0.48	0.52	0.47	0.53	0	0.05	0.95	0.92	0.08	0	1.0
SIP	0.59	0.41	0	0	0.41	0.59	0.44	0.56	0.42	0.58	0	0	1.0	0.89	0.11	0	1.0
SIB	—	—	—	—	—	—	—	—	0.43	0.57	0	0	1.0	0.64	0.36	0	1.0
BAR	0.40	0.60	0	0	0.19	0.81	0.23	0.77	0.31	0.69	0	0	1.0	1.0	0	0.08	0.92
SAN	0.19	0.81	0	0	0.35	0.65	0.20	0.80	0.17	0.68	0.15	0.31	0.69	0.88	0.12	0	1.0
MAN	0.22	0.78	0	0	0.40	0.60	0.08	0.92	0.19	0.54	0.27	0.02	0.98	1.0	0	0	1.0
GLO	0.02	0.98	0	0	0.44	0.56	0.37	0.63	0.10	0.40	0.50	0	1.0	1.0	0	0	1.0
KIT	0.19	0.81	0	0	0.33	0.67	0.02	0.98	0.10	0.60	0.30	0	1.0	1.0	0	0	1.0
PEI	0	0.41	0.57	0.02	0.15	0.85	0	1.0	0.51	0.34	0.15	0.01	0.99	0.92	0.08	0	1.0
BHA	0.37	0.63	0	0	0.31	0.69	0	1.0	0.10	0.57	0.33	0.14	0.86	1.0	0	0	1.0

DISCUSSION

Our main objective in these studies has been to assess the effect of varying levels of larval dispersal on the genetic structure of three littorinid species. Although we have no breeding data for the enzyme systems studies the patterns of electrophoretic banding appears to indicate the presence of first order allelic variation at three of the esterase systems described. The major result is that in those species endowed with limited dispersal capabilities (*L. saxatilis* and *L. obtusata*) one can find both geographic differentiation with respect to allele frequencies, and, in certain cases, alleles unique to a specific geographic region. In *L. littorea* a gastropod with extensive dispersal capabilities, owing to its lengthy pelagic period, no evidence was found for either significant regional differentiation, or region specific alleles. While it would be of additional interest to measure and compare allele frequency variance in these species, this method breaks down for extremely small values of one allele frequency, and, of course, becomes absolutely useless if there is monomorphism. Thus the low frequency alleles of *L. littorea* would tend to generate lower variance values (the predicted result) simply because of the low level of heterozygosity. Additional studies of polymorphic loci are needed, then, to eliminate this bias, and such studies are in progress.

Is the correlation between dispersal capability and the genetic differentiation of species unique to littorinids? Gooch, Smith and Knupp, 1972, have reported the results of a similar electrophoretic study for two gene-protein systems in 11 coastal populations of the prosobranch gastropod, *Nassarius obsoletus,* collected over a geographic range of 700 miles. This species like *L. littorea* has pelagic eggs and larvae, whose dispersal capabilities have been estimated to extend far over 250 miles, about one-fourth of its North American range. The results are unambiguous. For the diallelic LDH locus the range in frequencies of the major variant is 0.52–0.60, for the Pt locus, also diallelic, the frequency range of the major allele is 0.62–0.70. Thus the homogeneity of *Nassarius* populations is far more dramatic than even that of *L. littorea,* and indeed reflects the much broader dispersal range of *Nassarius,* compared to *L. littorea.*

The varying levels of genetic heterogeneity between populations may be interpreted in several ways. With limited dispersal (gene flow), one might argue, populations are afforded the opportunity to adapt genetically to the peculiar environmental conditions (both physical and biotic) in their locale. With local environmental conditions varying over the species range, one could anticipate an accompanying genetic differentiation. Alternatively, it is possible to suggest that isolation *per se* enhances the effect of random drift, serving effectively to promote genetic differentiation. Data on the distribution of gene frequencies cannot distinguish between these possibilities, for estimates of effective population size or actual dispersal rates have not been made in these species. It might be of interest, in distinguishing the effects of drift from selection, to examine many loci in these species and derive some estimate of the average individual heterozygosity. If random drift were effectively driving genes to fixation in species characterized by significant interpopulation heterogeneity then this should be reflected by low values of heterozygosity. Such a situation appears to be the case in the isolated Bogota population of *Drosophila pseudoobscura* (Prakash, Lewontin and Hubby, 1969), and in the marginal island populations of the field (mouse *Peromyscus polionotus* off the Florida coast (Selander, Smith, Yang, Johnson and Gentry, 1971). The selective model does not require this necessary condition.

In evolutionary terms the varying levels of larval dispersal in the three littorinid species may promote two additional effects. First, associated with the capability for extensive gene flow is the potential for widespread colonization. This is amply demonstrated by the recent and rapid appearance of *L. littorea* along the western Atlantic coast (Sumner *et al.*, 1911). The second, and closely related, effect associated with different levels of gene flow is that limited dispersal may promote genetic differentiation to the point of reproductive isolation. This appears to be true in gastropod molluscs where the greatest level of subspecies formation occurs in those forms showing the more restricted dispersal capabilities (Scheltema, 1971).

Dispersal rates, and the degree of gene flow between populations, are unknown for most species. For this reason, perhaps, these factors are often not considered explicitly in experimental studies of species variation. Nevertheless, it is clear that for the littorinids, and probably most prosobranch gastropods, mechanisms of dispersal may play a fundamental role in establishing the genetic structure of the species, and have a large impact on its evolutionary potential.

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SUMMARY

Gene-enzyme variation was examined, electrophoretically, at three non-specific esterase loci in 15 sympatric populations of the prosobranch gastropods: *Littorina littorea L. obtusata*, and *L. saxatilis*. Gene frequencies, determined by assuming a correspondence between bands on gels and alleles, revealed marked differences between the species. These differences appear to be correlated with the mechanisms and capabilities for larval disposal in these species.

LITERATURE CITED

Gooch, J., B. Smith and D. Knupp, 1972. Regional survey of gene frequencies in the mud snail *Nassarius obsoletus*. *Biol. Bull.*, **142**: 36–48.

Haseman, J., 1911. The rhythmical movements of *Littorina littorea* synchronous with ocean tides. *Biol. Bull.*, **21**: 113–121.

Hubby, J., and R. C. Lewontin, 1966. A molecular approach to the study of genic heterozygosity in natural populations. I. The number of alleles at different loci in *Drosophila pseudoobscura*. *Genetics*, **54**: 577–594.

Prakash, S., R. Lewontin and J. Hubby, 1969. A molecular approach to the study of genic heterozygosity in natural populations. IV. Patterns of genetic variation in central, marginal and isolated populations of *Drosophila pseudoobscura*. *Genetics*, **61**: 841–858.

Purchon, R. D., 1968. *The Biology of Mollusca*. [1st ed.] Pergamon Press, Oxford, England.

Scheltema, R., 1971. Larval dispersal as a means of genetic exchange between geographically separated populations of shallow-water benthic marine gastropods. *Biol. Bull.*, **140**: 284–322.

Selander, R., M. Smith, S. Yang, W. Johnson and J. Gentry, 1971. Biochemical polymorphism and systematics in the genus *Peromyscus*. I. Variation in the old-field mouse (*Peromyscus polionotus*). *Studies in Genetics VI., Univ. Texas Pub.*, **7103**: 49–90.

Sumner, F., R. Osborn and L. Cole, 1911. A biological survey of the waters of Woods Hole and vicinity. *Bull. Bur. Fish.*, 31: Section 3.

Reference: *Biol. Bull.*, **145**: 91–102. (August 1973)

VARIATION IN TEMPERATURE-SALINITY TOLERANCE BETWEEN TWO ESTUARINE POPULATIONS OF *PAGURUS LONGICARPUS* SAY (CRUSTACEA: ANOMURA)[1]

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Species living in an estuarine environment are normally subjected to frequent and variable fluxes in temperature and salinity. The hermit crab, *Pagurus longicarpus* Say, is found in the littoral area of estuaries from Nova Scotia to northern Florida, and from Sanibel Island, Florida, to Texas (Williams, 1965). The optimal salinity range for larval development through the megalopa stage is from 18.0 to 30.5‰ (Roberts, 1971). Optimal environmental conditions for growth, reproduction and survival of the postlarval stages, however, have not been clearly defined.

Tolerance defines the limits within which survival is possible (Alderdice, 1972). Fraenkel (1960) compared thermal tolerances of adult specimens of *P. longicarpus* collected from Woods Hole, Massachusetts, with two other intertidal zone inhabitants, *Limulus polyphemus* and *Littorina littorea.* Hermits could survive for one hour at 36° C, if allowed to recover for 24 hours at room temperature; 40° C was 100% lethal. More recently, Vernberg (1967) noted that *P. longicarpus* acclimated to 15° C survived longer than if acclimated to 5° C, when the environmental temperature was raised to 5, 10 or 15 degrees above the original acclimation temperature.

Kinne, Shirley and Meen (1963) found that the blood of *P. longicarpus* was isotonic with the environment at nine combinations of temperature and salinity. An increase in oxygen consumption as the salinity is lowered from approximately 18 of 5‰ (at 26 to 28° C) was reported for *P. longicarpus* by Nagabhushanam and Sarojini (1965). Vernberg and Vernberg (1972) investigated metabolic-temperature responses in latitudinally separated populations of the same species. Cold-acclimated crabs from Massachusetts and North Carolina populations consumed oxygen more rapidly than warm-acclimated animals. This apparent adaptational response was not observed in populations from Florida.

The euryhaline distribution of the adult and rather broad salinity tolerance of the larvae of *P. longicarpus* suggest that the species as a whole has the ability to adapt to most estuarine conditions. We investigated the tolerance of two populations from southern New Jersey to changes in salinity and temperature.

One population was collected from shallow flats at Grassy Sound Channel inside of Hereford Inlet on the Atlantic side of the Cape May peninsula (lat. 39°

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01.72′ north; long. 74° 48.10′ west), where the average annual salinity is about 30‰. The other, from an intertidal area along Delaware Bay south of Pierces Point on the Cape (lat. 39° 04.5′ north; long. 74° 54.9′ west), lived in mean salinities of about 20‰. The two populations are not in direct contact, although gene flow between them might occur by larval transport around the tip of Cape May at the ocean entrance of the Bay, a distance of 15–20 miles, or by a slightly shorter route through the Cape May Canal to the north of Cape May Point.

Preliminary experiments indicated a differential survival when Hereford and Cape Shore populations were subjected to various combinations of temperature and salinity. Hence, acclimation work was done in the laboratory to investigate whether differential survival was influenced primarily by acclimation to different field salinities, or whether more complex intraspecific differences might be indicated.

METHODS

Hermits were collected four times during the fall of 1971, and twice in early 1972. They were gathered by hand, with no intentional bias for size, though turbid conditions at the Cape Shore often necessitated groping.

Cape Shore field salinities are consistently lower and more variable than those at Hereford Inlet. Records for several years at the Hereford station show that the average annual salinity is approximately 30‰, with a range from 28.1–32.6‰. The average annual salinity at the Cape Shore is approximately 20‰, range 15–27‰ (H. H. Haskin, Rutgers University, personal communication).

Field collections were maintained in shallow communal trays in the laboratory for at least 1–2 weeks before they were used in experiments. Discrete collections were kept segregated. Hermits were regularly fed *Crepidula fornicata, C. plana* and chopped *Mercenaria mercenaria.* Feeding was discontinued one week before temperature-salinity tolerance was investigated.

Wet room maintenance temperatures ranged from 16–20° C. Normal maintenance salinity for the Hereford population was 30 ± 1‰, and 22 ± 2‰ for the Cape Shore population. Water was changed as often as necessary, but at least once a week. If sufficient natural seawater was available, it was used for water changes, but appropriate dilutions of commercially-available sea salts (Instant Ocean, by Aquarium Systems, Inc.) were also used for these changes and for all experimental salinities. Salinity determinations were performed using a modification of Harvey's $AgNO_3$ titration method (Welsh and Smith, 1953).

Ten pairs of trials compared the tolerance of the two geographic populations. In each, six hermits were used per temperature-salinity combination. Hermits were isolated in 50 ml of artificial seawater in individual 51 × 43 × 39 mm compartments in covered clear plastic boxes. The sex ratio in a group of six crabs depended on the availability of sexes in animals randomly removed from the shells prior to a trial. When possible, three of each sex were used. Crabs were induced to leave their gastropod shells by touching the tip of a soldering iron to the apex of the shell and allowing heat to drive the crab into a dish of cool water held directly beneath. Hermits were maintained in individual compartments for at least 3 days after removal, and those obviously injured were not used as experimental animals.

Hermits used in tolerance determinations were preserved after 96 hours and new crabs selected for the following trial. Length of the subcordate anterior shield was used as a size index (Markham, 1968).

Four temperatures and nine salinities were used to characterize temperature-salinity tolerance (Table I). Hermits from Hereford and Cape Shore fall collections maintained at their respective field salinities were used in Trials 1–4. In the first two trials, hermits were subjected to salinities from 5–25‰. In Trials 3 and 4, hermits were placed in salinities higher than 25‰. In Trials 5–7, we changed the natural acclimation salinity of 453 randomly-selected Hereford and Cape Shore crabs. Cape Shore crabs were held at 30‰, and Hereford at 22‰ for two weeks prior to determination of temperature-salinity tolerance. Trial 8 compared survival of 192 Cape Shore crabs acclimated to 30‰ for periods of one week and two months (not included in Table I).

Table I

Temperature-salinity combinations at which tolerance of Pagurus longicarpus *was investigated, and number of crabs used in each environmental combination (grand total = 1317).*

Temp °C	Salinity ‰								
	5.5	10.2	15.5	20.0	25.1	30.4	35.8	40.9	46.3
Cape Shore normal-acclimated to 22‰ (480 crabs: Trials 1, 2, 3, 4, 7)									
4	18	18	18	12	6	6	6	18	18
15	18	18	18	12	6	6	6	18	18
20	18	18	18	12	6	6	6	18	18
31	18	18	18	12	6	6	6	18	18
Hereford normal-acclimated to 30‰ (384 crabs: Trials 1, 2, 3, 4)									
4	12	12	12	12	12	6	6	12	12
15	12	12	12	12	12	6	6	12	12
20	12	12	12	12	12	6	6	12	12
31	12	12	12	12	12	6	6	12	12
Cape Shore acclimated to 30 ‰ (273 crabs: Trials 5, 6, 7)									
4	18	18	12	—	—	—	—	12	12
15	18	18	9	—	—	—	—	12	12
20	12	12	12	—	—	—	—	12	12
31	18	18	12	—	—	—	—	12	12
Hereford acclimated to 22‰ (180 crabs: Trials 5, 6)									
4	12	12	12	—	—	—	—	6	6
15	12	12	6	—	—	—	—	6	6
20	12	12	6	—	—	—	—	6	6
31	12	12	12	—	—	—	—	6	6

Trials 9 and 10, using winter-collected animals, attempted a more precise resolution of salinity tolerance limits in crabs maintained at their respective field salinities. Hermits at 15° C were subjected to salinities of 5.0, 7.5, 10.0, 12.5, 15.0, 17.5, 35.0, 37.5, 40.0, 42.5 and 45.0‰.

At the start of each 4-day trial, crabs were abruptly subjected to the appropriate acute conditions. Survival was then checked at 1-hour intervals for the first 5 hours, again at 9 and 12 hours, and thereafter at 12-hour intervals until the end of the 4-day period. The relative activity and response of each survivor to stimuli was also noted at each time check, as an index of morbidity.

Onset of morbidity was characterized by a loss of normal coordination and equilibrium. Crabs under greater stress showed decreased locomotory activity. As death approached, regular gill bailer action ceased and the maxillipeds protruded. The abdominal area often became transparent, especially in low salinities.

Any animals showing relatively sustained motion of appendages such as gill bailer, pereiopods, pleopods or antennae, when lightly stimulated with a blunt probe, were regarded as being alive. Only when further irritability to moderate probing of the abdomen or ventral body surface ceased, was an animal considered dead. As a precautionary measure, the animal was not removed from its compartment until mortality was confirmed at the next time check.

Crabs surviving the 4-day period would generally survive for several additional days. Tolerance estimates in this study are based on cumulative per cent mortality by 96 hours, pooled for each set of hermits in Table I, and computer fitted to generate response surfaces (Figs. 1–4).

The surface fitted is best described by the quadratic form employed by Box and Youle (1955) and reviewed by Alderdice (1972): $Y = B_0 + B_1X_1 + B_2X_2 + B_{11}X_1^2 + B_{22}X_2^2 + B_{12}X_1X_2$, where Y is arcsin square root of cumulative per cent mortality, X_1 is temperature in °C, X_2 is salinity in ‰, B_0 a mean effect, B_1 a linear effect of temperature, B_2 a linear effect of salinity, B_{11} a quadratic effect of temperature, B_{22} a quadratic effect of salinity, and B_{12} an interaction effect of temperature and salinity. B-values were calculated from the experimental points and pooled observed mortality by the method of least squares. A separate equation was obtained for each acclimation condition.

Translation of the center of a response surface depicts a change in tolerance. Rotation of the principal axes of regression so that they are no longer parallel to those of temperature and salinity implies temperature-salinity interaction (Alderdice, 1972). Elongation of response isopleths (plasticity) occurs along the axis of the factor exhibiting the lesser effect.

RESULTS

Mean size of P. longicarpus

Mean size of males from both Hereford Inlet and the Cape Shore was in all collections significantly larger ($\alpha = 0.01$) than the mean size of the respective females. Moreover, mean size of either sex of Hereford crabs was larger than mean size of animals of the same sex in each Cape Shore collection.

Estimation of optimal temperature-salinity combinations

The fitted response surfaces show regions of temperature and salinity where cumulative mortality at the end of 96 hours does not exceed 10% (Figs. 1–4). Normal-acclimated Cape Shore crabs show less than 10% cumulative mortality from 10–22° C, over a salinity range of 15–35‰ (Fig. 1). The response optimum

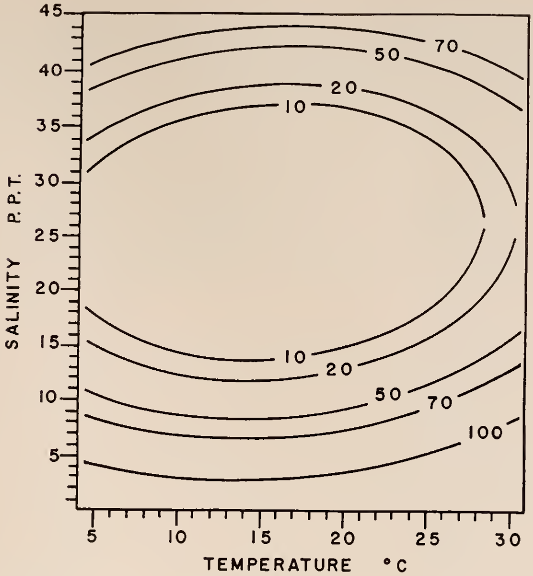

FIGURE 1. Estimation of per cent mortality of Cape Shore hermits acclimated to a normal habitat salinity of 22‰ based on fitted response surface to observed mortality at 96 hours under thirty-six conditions of temperature and salinity $(Y = 2.31695 - 0.063953X_1 - 0.151087X_2 + 0.002455X_1^2 + 0.003134X_2^2 - 0.000483X_1X_2)$.

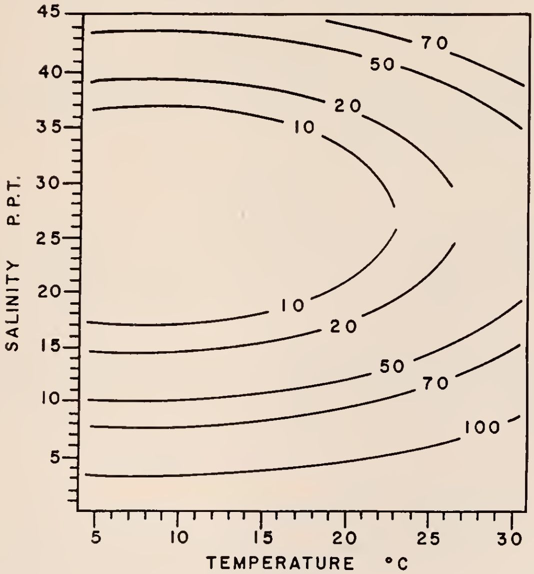

FIGURE 2. Estimation of per cent mortality of Cape Shore hermits acclimated to a higher than normal salinity of 30‰ based on fitted response surface to observed mortality at 96 hours under twenty conditions of temperature and salinity $(Y = 1.96682 - 0.016500X_1 - 0.136434X_2 + 0.001089X_1^2 + 0.002557X_2^2 - 0.000038X_1X_2)$.

should be located at the intersection of the geometric axes of the regression surface. Thus, optimum temperature for normal-acclimated Cape Shore crabs may be estimated to be 16° C, and optimum salinity at 25‰. Optimum conditions may be similarly found for each of the remaining three acclimation groups (Figs. 2–4).

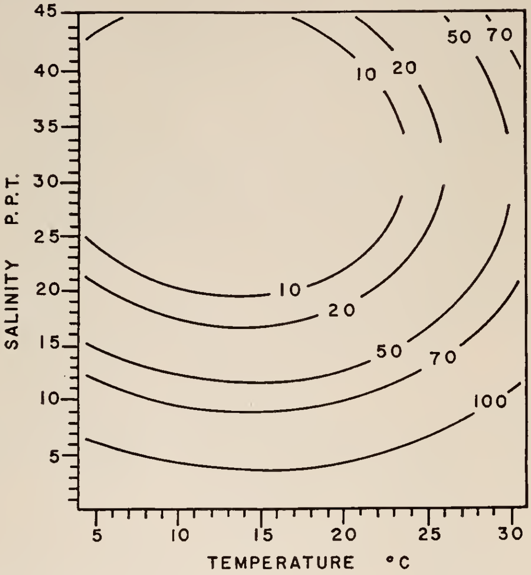

FIGURE 3. Estimation of per cent mortality of Hereford hermits acclimated to a normal habitat salinity of 30‰ based on fitted response surface to observed mortality at 96 hours under thirty-six conditions of temperature and salinity ($Y = 2.48732 - 0.079378X_1 - 0.12078X_2 + 0.002578X_1^2 + 0.001769X_2^2 + 0.000414X_1X_2$).

Effect of acclimation on temperature-salinity tolerance

When Cape Shore crabs were acclimated for two weeks to a higher than normal salinity of 30‰, the primary effect was a decreased high temperature survival (Figs. 1 and 2). Conversely, Cape Shore crabs acclimated to this high salinity realized better low temperature survival than crabs maintained in a normal habitat salinity. The response surface isopleths for Cape Shore animals acclimated to a higher than normal habitat salinity translated left almost 6° C along the temperature axis (Figs. 1 and 2). Despite euryplastic temperature tolerance, populations from the Cape Shore retained a salinity optimum relatively unresponsive to changes in acclimation salinity (Figs. 1 and 2).

Similar but less marked translations in temperature tolerance were observed in Hereford crabs acclimated to high and low salinity. In Hereford populations, however, the position of optimum salinity was obviously influenced by acclimation salinity (Figs. 3 and 4).

If temperature tolerance of Cape Shore crabs is now compared with Hereford crabs (Figs. 1 and 3), Cape Shore crabs showed wider temperature tolerance than their Hereford counterparts. When acclimated to the same salinity, variation in temperature tolerance between Cape Shore and Hereford crabs decreased, but Cape Shore crabs still survived wider extremes of temperature than did identically acclimated Hereford crabs. Differential temperature tolerance is more pronounced at 22‰ acclimation (Figs. 1 and 4) than at 30‰ (Figs. 2 and 3).

In interpreting temperature tolerance, one must keep in mind that a population acclimated to higher salinity will survive better at high salinity extremes, regardless of the temperature. Conversely, at any combination of temperature and low salinity, a low salinity acclimated group should have a survival advantage. Interaction of temperature and salinity is minimal and independent of acclimation salinity.

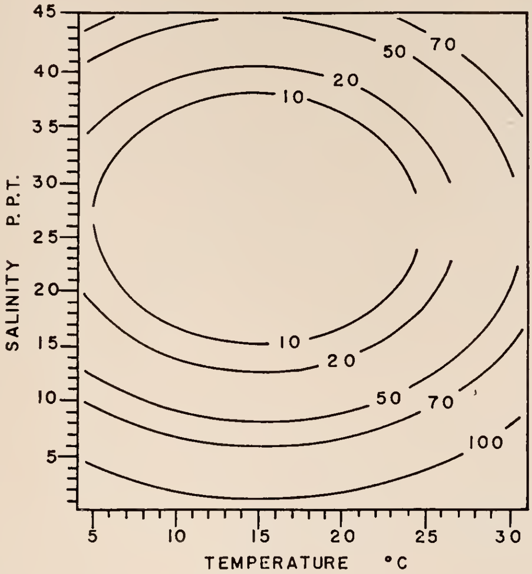

FIGURE 4. Estimation of per cent mortality of Hereford hermits acclimated to a lower than normal salinity of 22‰ based on fitted response surface to observed mortality at 96 hours under twenty conditions of temperature and salinity ($Y = 2.302 - 0.089643X_1 - 0.120942X_2 + 0.002916X_1^2 + 0.02255X_2^2 + 0.000112X_1X_2$).

Finally, independent of changes in acclimation salinity, low temperatures near 4° C represented more favorable conditions for *P. longicarpus* than high temperatures near 31° C.

Additional factors and their effects

Seasonal variation in time to reach LD-50 was negligible in both optimum and extreme temperature-salinity combinations, and seldom varied by more than 12 hours when populations collected at different times during 1971–72 were subjected to any of the temperature-salinity combinations.

Cape Shore crabs with only one week acclimation to higher than normal salinity showed tolerance intermediate between that of a normal-acclimated popula-

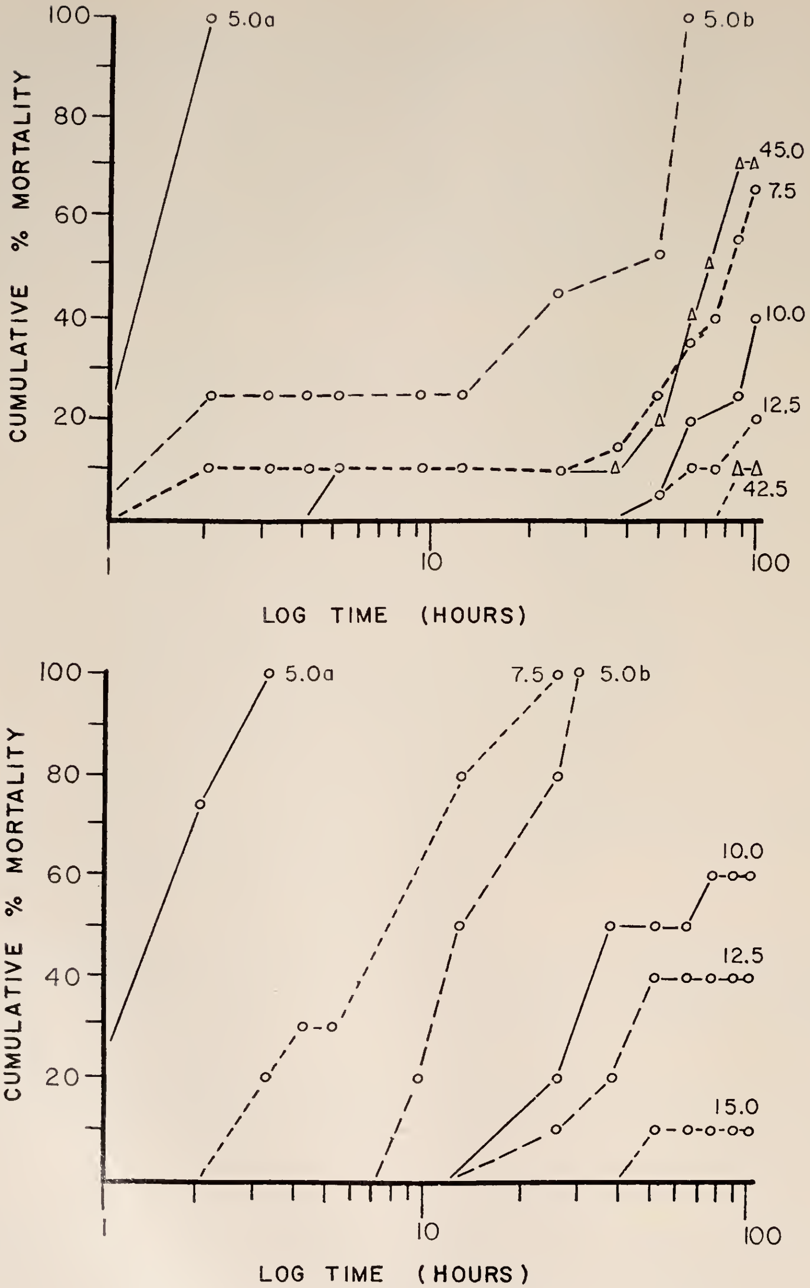

FIGURE 5. Cumulative per cent mortality of Cape Shore hermits acclimated to a normal habitat salinity of 22‰ by 96 hours at 15° C. Only those salinities in which mortality occurred are plotted (see text) (5.0*a* = acute transfer to 5.0‰; 5.0*b* = 96-hour acclimation to 15‰ before transfer to 5.0‰).

tion and groups held for two weeks under higher than normal salinity. There were no consistent differences in cumulative mortality after 96 hours between two-week and two-month acclimated groups.

Neither sex nor size appeared to influence mortality in *P. longicarpus,* although both in general often influence tolerance (Alderdice, 1972). In all trials, roughly equal percentages of each sex remained at LD-50, and when the mean size of living *versus* dead crabs at 96 hours was computed, ratios approached unity in groups of more than ten crabs.

Although no obvious selection for size or sex was noted, mortality seldom occurred at random throughout a 96-hour period. Rather, in both Hereford and Cape Shore populations, a large increase in cumulative mortality often occurred within a short time, especially at extremes of salinity (Figs. 5 and 6).

At less harsh salinity conditions, animals often showed some initial mortality, followed by increased tolerance, which caused leveling off of cumulative mortality (Fig. 5) as a degree of resistance adaptation was acquired (Kinne, 1970). Often, a second critical period would then be reached during which cumulative mortality increased rapidly until LD-100 was reached.

When salinity transitions are less abrupt than those employed in the present study, the nature of the response surfaces may be altered. For example, broad but gradual changes in salinity may postpone time to LD-50. A postponement of LD-50 was noted when animals were acclimated for four days to 15‰ before being transferred to 5% (Figs. 5 and 6). Note that LD-100 was nevertheless reached in both cases.

Cumulative mortality at 48 and 96 hours

Tolerance estimates for adult specimens of *P. longicarpus* have been somewhat arbitrarily based on cumulative mortality at 96 hours. A somewhat shorter time period might have been chosen, since most of the mortality usually occurred by 48 hours. For hermits from either population, areas of temperature and salinity enclosed by a 20% cumulative mortality isopleth were only slightly more compressed at the end of 96 hours. In experiments with a shrimp, *Penaeus aztecus,* Zein-Eldin and Aldrich (1965) demonstrated that it showed a degree of tolerance only slightly less over periods of 28 days when compared to 24 hours.

DISCUSSION

Roberts' (1971) estimates of salinity tolerance in the larval stages of *P. longicarpus* cover a period from hatching through the megalopa stage. When supplemented by estimates of adult tolerance proposed in the present study, they allow prediction of an optimum salinity range for most life-history stages. Ovigerous females used in Roberts' study were collected from a beach at Gloucester Point, Virginia. Larvae were hatched and maintained at 20° C in water of 20‰, and development at the same temperature followed at 10, 15, 20, 25 and 30‰.

FIGURE 6. Cumulative per cent mortality of Hereford hermits acclimated to a normal habitat salinity of 30‰ by 96 hours at 15° C. Only those salinities in which mortality occurred are plotted (see text) (5.0*a* = acute transfer to 5.0‰; 5.0*b* = 96-hour acclimation to 15‰ before transfer to 5.0‰).

Roberts concluded that tolerance of reduced salinity is the same for all four zoeal stages, and slightly less for the megalopae. Over a salinity range from 18.0–30.5‰, there were no significant differences in either zoeal mortality, hatching time, or intermolt duration. In comparison, over a 15–36‰ salinity range, normal-acclimated Cape Shore adults are estimated to show less than 10% mortality in a 96-hour period (Fig. 1). Hereford adults acclimated to the same low salinity show 10% mortality over a nearly equivalent 17–36‰ range (Fig. 4). If Virginia adults behave similarly, optimum salinity range at 20° C for adult specimens of *P. longicarpus* is apparently somewhat wider than optimum larval range. Larval development should be followed at salinities above 30‰, however, as this is an unlikely upper limit.

In the preceding discussion, one candidly assumed that tolerance of Virginia larvae could be compared with New Jersey adults acclimated to the same salinity. However, in the present study, adults from two New Jersey populations were acclimated for two weeks to the same salinity, and tolerances were not identical.

If the physiological basis for differential survival between adult Hereford and Cape Shore populations resided solely in the different natural-acclimation salinities of 22‰ and 30‰, equivalent acclimations would be expected to produce identical temperature-salinity tolerance. However, since acclimation of both populations to the same salinity consistently produced tolerance intermediate to that of natural-acclimated populations, more complex interactions or intraspecific physiological differences may determine tolerance.

The Cape Shore hermits inhabit a more extensive network of shallow flats and bars than is found at Hereford Inlet. Both temperature and salinity are more variable here. Proximity to fresh water sources influences salinity variation, while the shallow nature of the area can permit summer temperatures to rise above those of the more ocean-buffered system at Hereford Inlet. Water temperature may vary as much as 5° C during a summer tidal cycle (Hendler and Franz, 1971). In winter, Cape Shore crabs may experience lower temperatures since at least some are known to remain in the intertidal zone under winter conditions. In contrast, the Hereford population migrated completely out of the intertidal area, so that by January, none remained on exposed flats.

Because Cape Shore crabs are presumably subjected to seasonally wider extremes of temperature, a degree of selection for temperature resistant forms may occur in this population. Unless high temperature tolerance is normally present in a population, however, laboratory acclimation to a low salinity apparently will not increase temperature tolerance. In populations of Hereford crabs acclimated to 22‰, temperature tolerance was not markedly elevated.

Salinity-dependent temperature tolerance exhibited by Cape Shore crabs may derive from osmotic considerations. Crabs naturally-acclimated to low salinities, when subjected to higher than normal salinity acclimation stress, may be spending energy in hyporegulating, which makes them more susceptible to temperature stress. In support of this suggestion, preliminary unpublished investigations by one of us (D. C. B.) do indicate a narrow range of active osmoregulation in *P. longicarpus*. Blood of adults acclimated to 30‰ was demonstrably hypotonic in environmental salinities between 30 and 40‰.

Crabs acclimated to high salinity may find low salinity environments equally deleterious. Larvae reared at 20‰ and then placed into 10‰ showed significantly greater mortality than those placed into 30‰ (Roberts, 1971). Adults responded similarly. *P. longicarpus* increased its oxygen consumption in low salinities at a temperature of about 27° C (Nagabhushanam and Sarojini, 1965). This was interpreted as an increase in osmoregulatory work, although alternative explanations are possible, *e.g.*, increased locomotor activity (Lockwood, 1967, page 150), or scaphognathite activity. A European hermit crab, *Eupagurus bernhardus*, showed 15.4% swelling when subjected for one hour to a hypotonic environment. Swelling was subsequently relieved by increased rates of urine production (Davenport, 1972).

The response surfaces of *P. longicarpus* can be compared with those fitted for another decapod. Haefner (1969) used twelve different combinations of temperature and salinity to generate a response surface for the sand shrimp, *Crangon septemspinosa*. This species inhabits estuarine environments similar to those of *P. longicarpus*, and in fact, may be collected in the intertidal zone at the Cape Shore and Hereford Inlet. Price (1962) collected this shrimp throughout Delaware Bay in salinities which ranged from 4.4 to 31.4‰. Response surface isopleths show quite similar elongation and distribution, and salinity exerts a greater effect on tolerance than does temperature. As with *P. longicarpus*, lower or higher temperatures reduce the salinity range in which maximum survival can be expected.

Although a multifactor analysis was designed to study tolerance of *Crangon*, shrimp were collected from only a single geographical area out of the total species range, and no changes in acclimation salinity were attempted. As the present study has illustrated, estimates of tolerance made independently of acclimation work may be of limited value, and response surface models generated from such preliminary data should probably be applied only to populations naturally-acclimated to similar salinities.

We are indebted to P. H. Sutter, R. S. Lehman, and W. F. Tyndall, who aided in compiling and editing the computer program for fitting the response surface. We appreciate the comments of P. A. Haefner, Jr., on an early draft of the manuscript, and of J. M. Teal, who critically reviewed the final draft.

SUMMARY

1. A hermit crab, *Pagurus longicarpus* Say, was collected from two sites in southern New Jersey at approximately the same latitude but on different sides of the Cape May peninsula. Those from Hereford Inlet lived in mean salinity of 30‰, and Delaware Bay Cape Shore hermits lived in 20‰.

2. Adults from both populations were acclimated for two weeks to 22‰ and 30‰ and were then subjected to 20–36 combinations of temperature and salinity. Cumulative mortality after 96 hours was used to fit response surfaces for estimating tolerance.

3. Hereford and Cape Shore populations differed in temperature-salinity tolerance. Cape Shore crabs showed wider temperature tolerance than identically-

acclimated Hereford crabs, while the latter survived greater variation in salinity. Apparently, different acclimation salinities are not alone sufficient to explain differential tolerance.

4. Salinity tolerance of adults from New Jersey is compared with tolerance of zoeae and megalopae from previous studies in Virginia. At 20° C, zoeal salinity tolerance is identical from 18.0–30.5‰. At the same temperature, estimated optimal salinity range for adults is 15–36‰.

LITERATURE CITED

ALDERICE, D. F., 1972. Factor combinations. Pages 1659–1772 in O. Kinne, Ed., *Marine Ecology, Vol. I, Pt. 3.* Wiley-Interscience, New York.

BOX, G. P., AND P. V. YOULE, 1955. The exploration and exploitation of response surfaces: An example of the link between the fitted surface and the basic mechanism of the system. *Biometrics,* **11**: 287–323.

DAVENPORT, J., 1972. Volume changes shown by some littoral anomuran Crustacea. *J. Mar. Biol. Ass. U. K.,* **52**: 863–877.

FRAENKEL, G., 1960. Lethal high temperatures for three marine invertebrates: *Limulus polyphemus, Littorina littorea,* and *Pagurus longicarpus. Oikos,* **11**: 171–182.

HAEFNER, P. A., JR., 1969. Temperature and salinity tolerance of the sand shrimp, *Crangon septemspinosa* Say. *Physiol. Zool.,* **42**: 388–397.

HENDLER, G., AND D. R. FRANZ, 1971. Population dynamics and life history of *Crepidula convexa* Say (Gastropoda: Prosobranchia) in Delaware Bay. *Biol. Bull.,* **141**: 514–526.

KINNE, O., 1970. Temperature. Pages 321–514 in O. Kinne, Ed., *Marine Ecology, Vol. I, Pt. 1.* Wiley-Interscience. New York.

KINNE, O., E. K. SHIRLEY, AND H. E. MEEN, 1963. Osmotic responses of hermit crabs (*Pagurus longicarpus* Say) exposed to various constant temperatures and salinities. *Crustaceana,* **5**: 317.

LOCKWOOD, A. P. M., 1967. *Aspects of the Physiology of Crustacea.* W. H. Freeman and Co., San Francisco, 328 pp.

MARKHAM, J. C., 1968. Notes on growth-patterns and shell-utilization of the hermit crab *Pagurus bernhardus* (L.). *Ophelia,* **5**: 189–205.

NAGABHUSHANAM, R., AND R. SAROJINI, 1965. The influence of low salinity upon the oxygen consumption in the hermit crab, *Pagurus longicarpus* Say. *Sci. Cult.,* **31**: 43–44.

PRICE, K. S., JR., 1962. Biology of the sand shrimp, *Crangon septemspinosa,* in the shore zone of the Delaware Bay region. *Chesapeake Sci.,* **3**: 244-255.

ROBERTS, M. H., JR., 1971. Larval development of *Pagurus longicarpus* Say reared in the laboratory. II. Effects of reduced salinity on larval development. *Biol. Bull.,* **140**: 104–116.

VERNBERG, F. J., 1967. Some future problems in the physiological ecology of estuarine animals. Pages 554–557 in G. H. Lauff, Ed., *Estuaries.* Publication No. 83, American Association for the Advancement of Science, Washington, D. C.

VERNBERG, W. B., AND F. J. VERNBERG, 1972. *Environmental Physiology of Marine Animals.* Springer-Verlag, New York, 346 pp.

WELSH, J. H., AND R. I. SMITH, 1953. *Laboratory Exercises in Invertebrate Physiology.* Burgess, Minneapolis, 126 pp.

WILLIAMS, A. B., 1965. Marine decapod crustaceans of the Carolinas. *U. S. Fish. Wild. Serv., Fish. Bull.,* **65**: 1–298.

ZEIN-ELDIN, Z. P., AND D. V. ALDRICH, 1965. Growth and survival of postlarval *Penaeus aztecus* under controlled conditions of temperature and salinity. *Biol. Bull.,* **129**: 199–216.

Reference: *Biol. Bull.*, **145**: 103–118. (August 1973)

LATITUDINAL EFFECTS ON METABOLIC RATES IN THE FROG, *ACRIS CREPITANS:* SEASONAL COMPARISONS

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In his review of latitudinal effects on the physiological properties of animal populations, Vernberg (1962) points out that a variety of physiological differences occur among latitudinally separated populations of some species. The differences are of considerable interest both to the comparative physiologist and to the ecologist, since they provide insight into the relative roles of longer term genetic changes in populations *versus* shorter term physiological changes of organisms in their adaptation to varying climatic regimes. Differences in whole body oxygen consumption which are apparently genetically fixed have been reported for a variety of organisms. Where these differences have been reported, they are such as to suggest that latitudinal compensation in metabolism for temperature occurs. In general, northern populations have a higher rate of oxygen consumption than southern populations of the same or related species when determined over a wide range of temperatures (Bullock, 1955; Prosser, 1955; Vernberg, 1962; Tashian and Ray, 1957; Hutchison, Whitford and Kohl, 1968).

Several investigators have compared the metabolic rates of latitudinally separated populations of the same species of anuran. Tashian and Ray (1957) compared northern and southern subspecies of *Bufo boreas* and found no significant differences between them. Jameson, Taylor and Montjoy (1970) however, report a great deal of variability in metabolic rates among populations of the frog, *Hyla regilla*. Dunlap (1972) reports that for warm acclimated *Acris crepitans,* South Dakota frogs have higher metabolic rates at low temperatures than do Texas frogs.

When comparing latitudinally separate populations one would hope to distinguish between short-term, environmentally induced variations in metabolic patterns and those that are genetically determined. However, the occurrence of marked seasonal changes in the metabolic rates of intact frogs which has been reported for a number of species, *e.g., Rana fusca* (Krogh, 1904); *R. esculenta* (Stangenberg, 1955); *R. pipiens* (Fromm and Johnson, 1955); *R. temporaria* (Dolk and Postma, 1927); *Acris crepitans* (Dunlap, 1969); *Hyla regilla* (Jameson *et al.,* 1970), can complicate the interpretation of comparative studies. That these changes are not due to short-term thermal acclimation to seasonal differences in temperature is evidenced by the fact that recent investigators who have reported the seasonal changes have compared animals acclimated at the same temperatures (Stangenberg, 1955; Dunlap, 1969; Jameson *et al.,* 1970).

There is little organized information available on the latitudinal effects on seasonal shifts in metabolic rates. However, in many Amphibia, including *Acris crepitans,* southern populations have a longer breeding season and a shorter period of dormancy than more northern populations (Stebbins, 1951). If the seasonal metabolic changes are related to the periods of reproduction and dormancy, one could expect that the seasonal metabolic cycles of latitudinally separated populations would be out of phase with each other. When thermally acclimated samples of latitudinally separated populations are compared at some point in time and differences found, one cannot determine whether the observed differences are due to differences in the genotypes of the populations or simply to the comparison of two non-homologous points in a seasonal metabolic cycle in the acclimatized (*sensu* Prosser, 1958) populations. Conversely, the seasonal differences could conceivably mask genetically controlled differences between the populations.

Consequently, in view of the paucity of information concerning latitudinal effects and latitudinal-seasonal interactions on the metabolic rates of anurans, a comparison was made of the seasonal changes in the metabolic rates of acclimated cricket frogs from two latitudinally widely separated populations. The results were then compared with information on seasonal activities and environmental and body temperatures to see if there were correlations between metabolic patterns and the environmental temperatures which the frogs normally encounter.

Materials and Methods

Samples of cricket frogs were collected near Vermillion, South Dakota (Latitude 42° 48′ N; elevation 1220 ft) and Austin, Texas (Latitude 30° 18′ N; elevation 615 ft). Vermillion lies approximately 870 miles north of Austin.

Frogs from South Dakota were collected at intervals between April and early November, 1967 (Fig. 1), one to two days prior to placing them in the acclimation chambers. Texas frogs were collected at intervals between October and December, 1968, and between early April and early July, 1969 (Fig. 2). The Texas frogs were shipped to Vermillion *via* air express and were placed in the acclimation chambers upon arrival.

Groups of frogs were acclimated in the dark for 5–7 days (Dunlap, 1969) at temperatures of 5, 15 or 25 ± 1° C. They were maintained in loosely covered glass jars and had access to free water but were not fed during the course of acclimation.

Oxygen consumption was measured at 15° C for individual frogs using a refrigerated Gilson differential microrespirometer equipped with 100 ml flasks. Each flask received 5 ml deionized water in the animal chamber and carbon dioxide was absorbed by 1.5 ml of 20% KOH placed in the side arm. The flasks were equilibrated for 30 min and readings were taken every 15 min for 2 hr. Stability was routinely monitored by the insertion of a blank specimen vessel.

Metabolic rates are given as μl/g per hr STP of oxygen and are based on the average hourly uptake over the 2 hr period. These can best be considered as routine rates in the sense of Fry (1957) inasmuch as there was no control of spontaneous locomotor activity during the 2 hr period of determination (Dunlap, 1971).

Animals collected at a particular time from each locality were assigned to the three acclimation temperatures on a random basis with the restrictions that there should be approximately equal proportions of males and females and equal proportions of three arbitrarily assigned size classes within each acclimation group. I was, however, unable to control size variation between samples taken at different times of the year. Furthermore, South Dakota frogs averaged considerably larger

(1.27 g) than the Texas frogs (0.90 g). Consequently, comparisons between samples from different times of the year or between samples from the two localities were made by direct comparison of metabolism-body weight regression lines following a log-log transformation of the data. The statistical techniques used in the analyses are from Li (1957) and Ostle (1963).

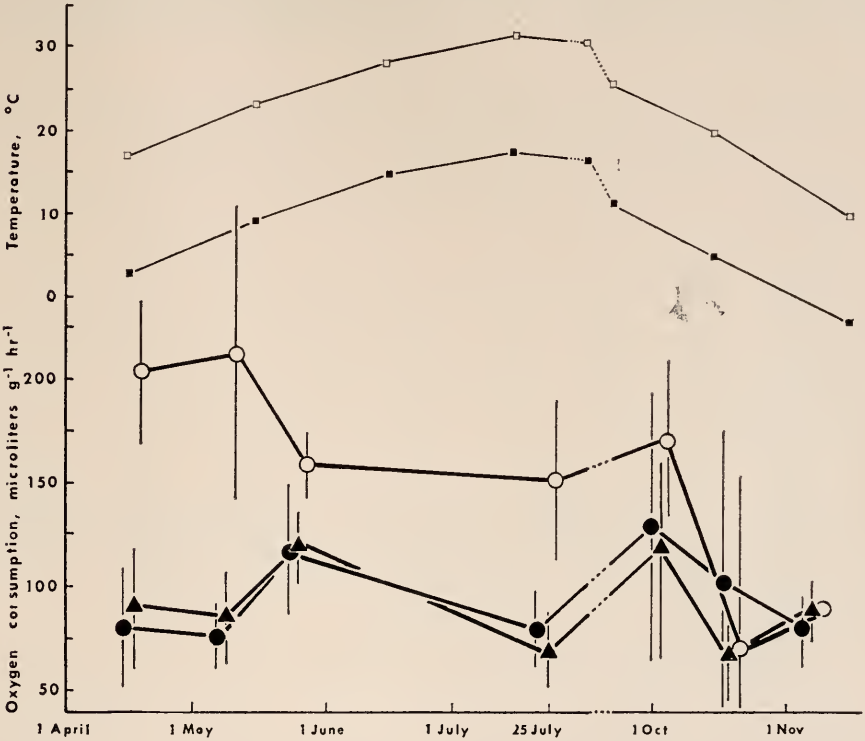

FIGURE 1. The three lower curves represent routine oxygen consumption in *Acris crepitans* from South Dakota at various times of the year determined at 15° C following acclimation of the frogs at 5, 15 and 25° C. Each point on the graph represents the predicted mean metabolic rate for frogs weighing 1.25 grams. The vertical lines represent the 95% confidence intervals for the predicted means. Symbols representing the acclimation temperatures are 5° C (filled circle); 15° C (filled triangle); 25° C (open circle). The upper pair of lines represent the mean maximum monthly temperatures (open square) and the mean minimum monthly temperatures (filled square) at Vermillion, S. D.

RESULTS

Mean metabolic rates of specimens of *Acris crepitans* acclimated at 5, 15 or 25° C and determined at 15° C at different times of the year are shown in Figures 1 and 2. With the exception of the mean metabolic rate of frogs acclimated at 25°C and determined on 6 November, which rate is based on only two animals, the means for the South Dakota frogs (Fig. 1) are based on samples of 5–7 animals. In all, these data are based on a total of 121 different frogs. The means for the Texas specimens of *Acris* (Fig. 2) are based on 6–8 frogs except for the animals acclimated at 5° C and determined 8 April, 29 April, 4 October and 1 November which means are based on samples of 4–5 frogs. The Texas data are based on a total of 117 animals.

Since there was marked variation in the mean body weight of samples of frogs collected at different seasons, the mean metabolic rates shown in Figures 1 and 2 are corrected for body weight. The values for the South Dakota frogs shown in Figure 1 are calculated for frogs of 1.25 g body weight; for the Texas frogs the corrected means are for frogs of 1.00 g body weight. The 95% confidence intervals of the means are also shown in Figures 1 and 2. It should be borne in mind, however, that the variation in the width of the confidence intervals is due not only to the variation among variances of the samples but also to the variable sample size and to seasonal differences in the degree of deviation of mean body weights of the samples from the overall mean.

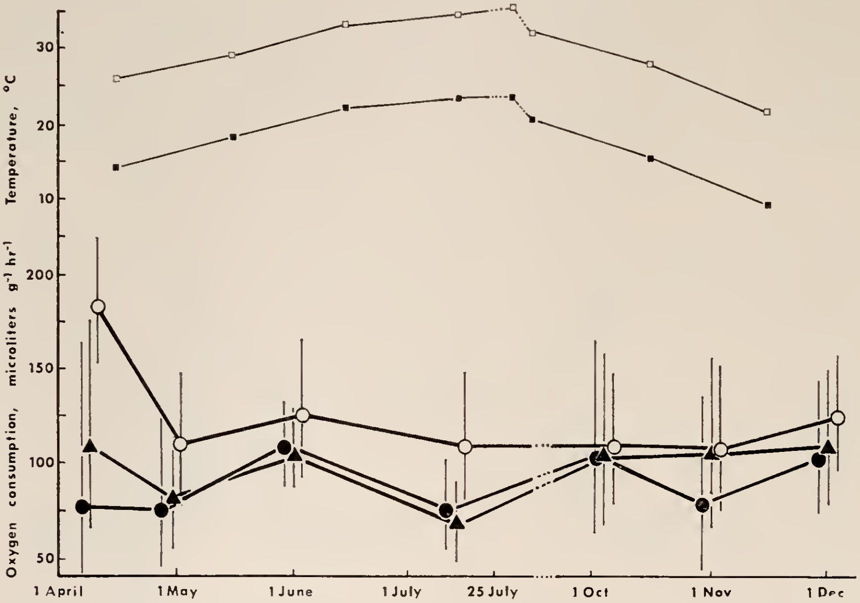

FIGURE 2. The three lower curves represent routine oxygen consumption in *Acris crepitans* from Texas at various times of the year determined at 15° C following acclimation of the frogs at 5, 15 and 25° C. Each point on the graph represents the predicted mean metabolic rate for frogs weighing 1.0 grams. The vertical lines represent the 95% confidence intervals for the predicated means. The upper pair of lines represent the mean maximum and mean minimum monthly temperatures at Austin, Texas. Symbols are as in Figure 1.

Acclimation effects

While there were marked differences in mean body size among samples collected on different dates, on any one date the samples were stratified with respect to body weight and sex. Consequently, Student's t-values were calculated for all pairs of means on any one sample date. The results are shown in Table I. As may be seen, there is no statistically significant difference between the means of samples acclimated at 5 and 15° C ($P > 0.05$) on any date for either the South Dakota or Texas series.

No significant differences were found among the means of the three acclimation groups from October into December ($P > 0.05$) except for Texas frogs acclimated at 25 and 5° C in November ($P < 0.05$).

TABLE I

Student's t-values comparing the mean metabolic rates of samples of Acris crepitans *from South Dakota and Texas which had been acclimated at 5, 15 and 25° C and determined at 15° C on the dates indicated. The degrees of freedom for each calculated t-value is enclosed in parentheses.*

Locality	Temperatures compared	Date of determination						
		16 April	7 May	27 May	24 July	2 Oct	18 Oct	6 Nov
South Dakota	25–15° C	6.715** (10)	4.598** (10)	7.140** (10)	4.840** (10)	1.938 (10)	1.527 (8)	0.426 (7)
	25–5° C	7.356** (10)	5.362** (10)	8.590** (10)	4.674** (10)	0.851 (11)	0.798 (9)	0.334 (7)
	15–5° C	0.691 (10)	1.926 (10)	1.509 (10)	0.342 (10)	1.204 (9)	2.850 (9)	1.248 (12)
		8 April	29 April	31 May	20 July	4 Oct	1 Nov	3 Dec
Texas	25–15° C	3.08* (9)	2.936* (10)	1.78 (14)	4.557** (10)	1.16 (11)	0.045 (11)	2.08 (10)
	25–5° C	3.24* (8)	3.860** (9)	1.11 (12)	3.420** (10)	0.65 (9)	2.73* (8)	1.68 (11)
	15–5° C	2.02 (7)	0.412 (9)	0.617 (12)	1.898 (10)	1.44 (8)	1.54 (9)	0.367 (11)

* $P < 0.05$.
** $P < 0.01$.

From April through July the South Dakota frogs acclimated at 25° C had metabolic rates which were consistently higher than and significantly different from ($P < 0.01$) either the 5 or 15° C acclimation groups. With the exception of the samples of 31 May ($P > 0.05$), the metabolic rates of Texas frogs acclimated at 25° C were higher than and significantly different from ($P < 0.05$) those acclimated at 5 and 15° C in April through July.

Seasonal effects

In an effort to determine whether there are significant seasonal variations among samples from one locality, the metabolic data from samples of frogs which had been acclimated at the same temperature were compared. Inasmuch as the mean body weights of the samples varied considerably among the different sampling periods and since the metabolic rate may be a size-dependent variable (Dunlap, 1969, 1971) in these frogs, regression equations were calculated following a log-log transformation of the data and were compared. The initial hypothesis being tested is that one regression line can be used for all seven sets of seasonal data (Ostle, 1963). If the hypothesis is rejected, the hypothesis of equality of the slopes (regression coefficients) of the lines is tested. If the latter hypothesis is accepted, the adjusted means of the sample can be compared using an analysis of covariance. The results of these tests are shown in Table II. As may be seen, only in the series of Texas samples acclimated at 5° C can all seven sets of data be represented by one regression line ($P > 0.25$). Hence, this series of samples shows no statistically significant seasonally related changes in metabolic rate. In all the other sets, the hypothesis of one regression line being adequate for all is rejected ($P < 0.005$). Furthermore, in none of these are the slopes significantly different ($P > 0.05$) and, as might be expected, in none of these are the size adjusted means all equal ($P < 0.005$).

TABLE II

Comparison of the regression lines of routine metabolic rate for samples of Acris crepitans *acclimated at 5, 15 and 25° C and determined at 15° C at different times of the year. For the Texas frogs, the samples being compared for each acclimation temperature were determined on 8 April, 29 April, 31 May, 20 July, 4 Oct., 1 Nov. and 3 Dec. For the South Dakota frogs the dates are 16 April, 7 May, 27 May, 24 July, 2 Oct., 18 Oct. and 6 Nov.*

Locality	Hypothesis being tested	Acclimation temperature								
		5° C			15° C			25° C		
		F-ratio	d.f.	P	F-ratio	d.f.	P	F-ratio	d.f.	P
Texas	One regression line?	1.268	12, 24	>0.25	3.336	12, 32	<0.005	3.314	12, 33	<0.005
	Slopes equal?	—	—		2.021	6, 32	>0.05	2.085	6, 33	>0.05
	Adjusted means equal?	—	—		4.026	6, 38	<0.005	3.899	6, 39	<0.005
South Dakota	One regression line?	4.546	12, 28	<0.001	3.433	12, 27	<0.005	3.938	10, 26	<0.005
	Slopes equal?	1.827	6, 28	>0.10	1.928	6, 27	>0.10	0.494	5, 26	>0.50
	Adjusted means equal?	6.331	6, 34	<0.001	4.200	6, 33	<0.005	8.050	5, 31	<0.0005

The regression lines within each series were compared pair by pair to determine which samples were significantly different from each other. The results may be summarized as follows.

In the series of frogs from Texas which had been acclimated at 15° C, the mean metabolic rate of the sample of 20 July was significantly different from ($P < 0.05$) and lower than those of all other samples except the samples of 29 April and 4 October. For the Texas series which had been acclimated at 25° C, the mean metabolic rate of the sample of 8 April was significantly different from ($P < 0.05$) and higher than those of all other samples with the exception of the samples of 31 May and 3 December.

In the South Dakota series of frogs which had been acclimated at 5° C, the mean metabolic rates of the samples of 2 and 18 October were significantly different from ($P < 0.05$) and higher than those of all the others with the exception of the sample of 27 May. For the frogs acclimated at 15° C, the mean rates of the 2 October and the 27 May samples were significantly different from ($P < 0.05$) and higher than those of samples determined on 18 October and 27 July, but were not significantly different from the other samples. For frogs acclimated at 25° C, the mean metabolic rates of the samples of 16 April and 7 May were significantly different from ($P < 0.05$) and higher than than those of any of the other samples except that of 2 October. The mean rate of the latter sample, in turn, was significantly different from ($P < 0.05$) and higher than those of the samples of 18 October and 6 November but did not differ significantly from the samples of 27 May and 24 July ($P > 0.05$).

Locality effects

Comparisons of metabolism-body weight regression equations calculated following a log-log transformation of the data were made for Texas and South Dakota samples of *Acris* acclimated and determined at approximately the same time of year. Samples were compared for the following six dates; the first date in each case represents the Texas sample: 8, 16 April; 29 April, 7 May; 31, 27 May; 20, 24 July; 4, 2 October; 1, 6 November. Since the metabolic rates of frogs acclimated at 5 and 15° C and determined at any one time were not significantly different from each other for either the Texas or South Dakota animals, the regression lines of the four sets of samples (5 and 15° C acclimated frogs from both Texas and South Dakota) from each of the dates were compared. In none of the comparisons could the hypothesis that one regression line can be used for all four samples be rejected ($P > 0.05$). In only two of the sets was $P < 0.10$. These were the October comparisons in which the South Dakota samples tend to have a higher metabolic rate (120 μl/g per hr) than the Texas samples (100 μl/g per hr) and the November samples in which the metabolic rates of frogs from both localities average somewhat lower when acclimated at 5° C than when acclimated at 15° C. No significant differences between the regression lines were found for the 25° C acclimated frogs in the 8, 16 April ($P > 0.10$); 31, 27 May ($P > 0.25$) or 1, 6 November ($P > 0.25$) samples. The warm acclimated Texas and South Dakota samples were significantly different for the 29 April, 7 May ($P < 0.0005$), July ($P < 0.025$) and October ($P < 0.05$) comparisons with the mean metabolic rates of the South Dakota samples being the higher.

Metabolic rates of Texas frogs determined at 25° C

Early April, late April and early December samples of Texas frogs which had been acclimated at 5, 15 and 25° C were determined at 25° C as well as at 15° C and the means of the different acclimation groups on each determination date were compared using Student's t-test. The mean metabolic rate for each treatment of samples determined at both 15 and 25° C is shown in Figure 3. The purpose of the determinations at 25° C was to provide additional information on possible seasonally related changes in acclimation pattern and Q_{10} values of the frogs. As was shown earlier (Table I), statistically significant acclimation effects are apparent in April frogs when the metabolic rates are determined at 15° C. At that temperature frogs acclimated at both 5 and 15° C are significantly different from frogs acclimated at 25° C but not from each other. However, when both sets of April samples are determined at 25° C, only the frogs acclimated at 5° C differ significantly from the 25° C frogs ($P < 0.02$). The metabolic rates of frogs acclimated at 15° C do not differ significantly from either the 5 or the 25° C samples ($P > 0.10$). As was true for the frogs determined at 15° C, no significant acclimation effects can be demonstrated for the December samples determined at 25° C ($P > 0.10$) although the metabolic rates of the frogs acclimated at 25° C average higher than those acclimated at 5 or 15° C. Thus the pronounced inverse compensation shown at lower determination temperature is replaced by either a weak or no acclimation effect at 25° C.

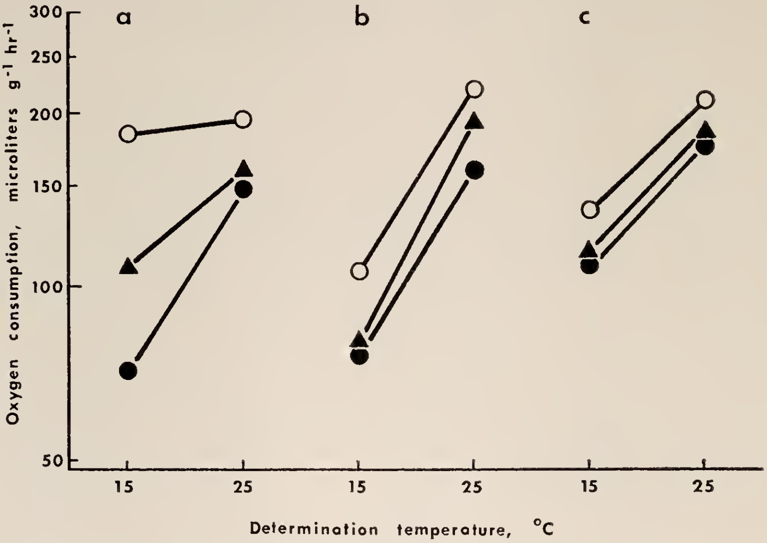

FIGURE 3. Routine oxygen consumption in *Acris crepitans* from Texas on three different sampling dates determined at 15 and 25° C following acclimation at 5, 15 and 25° C. Determinations were made on April 8–9 (a); April 29–30 (b); December 3–7 (c). Each point on the graph represents the mean metabolic rate of six frogs at the indicated determination temperature. Symbols are as in Figure 1.

Q_{10} values for the acutely measured rate-temperature (R-T) curves between 15 and 25° C shown in Figure 3 fall into three groups: low (1.1) for the 25° C frogs on 8–9 April; intermediate (1.5–1.6) for all three acclimation groups in December and the 15° C frogs in early April; and high (2.1–2.4) for all acclimation groups on 29–30 April and the 5° C frogs on 8–9 April. The 29–30 April values agree with July samples (Dunlap, 1972) in having Q_{10} values of greater than 2.0 between 15 and 25° C. Thus the Q_{10} values between 15 and 25° C for the acutely measured R-T curves tend to be relatively uniform and high for the late April and July frogs, uniform and intermediate for the December frogs and variable for the early April frogs. In the latter series, the Q_{10} values are inversely proportional to the acclimation temperature with the Q_{10} value of the warm acclimated frogs approximating 1.0. The Q_{10} values for the acclimated R-T curves between 15 and 25° C tend to be higher than those of the corresponding acute curves, averaging 2.8 in late April and July and 1.8 in early April and December.

DISCUSSION

Although the seasonal metabolic cycles of the two populations differ in detail, they do share a number of features in common. On any one date at each locality, no statistically significant differences could be demonstrated between the frogs acclimated at 5 and those acclimated at 15° C when both were determined at 15° C. Furthermore, the metabolic rates of frogs from Texas and South Dakota which had been acclimated at 5 and 15° C were statistically indistinguishable when measured at about the same time of year. The timing of the two small metabolic peaks on the seasonal curves for the 5 and 15° C frogs (Fig. 1 and 2) in late May and early October is similar for the two populations. For both populations, the metabolic rates of the warm acclimated spring and summer animals are higher than those of the frogs acclimated at 5 and 15° C when all are determined at 15° C. Consequently, the pattern of inverse compensation at 15° C reported for *Acris* in earlier papers (Dunlap, 1969, 1971 and 1972) persists throughout much of the spring and summer at both localities. Both populations also exhibit a pronounced early (8 April, Texas or 16 April, 7 May, South Dakota) spring peak in the metabolic rates of warm acclimated frogs.

Although there are no clearcut differences in the metabolic rates of the two populations for frogs acclimated at 5 and 15° C, the rates of warm acclimated frogs average higher for South Dakota frogs than for Texas animals. From late May to October for the South Dakota frogs and from late April to November for the Texas frogs, the metabolic rates of the warm acclimated frogs are essentially uniform averaging about 160 μl O_2/g per hr for the South Dakota frogs and about 110 μl O_2/g per hr for the Texas frogs. Earlier in the spring, however, rates are higher, averaging about 210 μl O_2/g per hr for the South Dakota frogs and 180 μl O_2/g per hr for the Texas animals. In the autumn, the metabolic rates of the Texas frogs remain constant or perhaps increase slightly. In the South Dakota frogs, on the other hand, the metabolic rates of all the samples decrease markedly after early October. The decrease is especially pronounced for the warm-acclimated animals and by late October and early November the metabolic rates reach the lowest levels found in this study. Although data are not available for the winter months, the pattern suggests that the metabolic rates of the warm acclimated Texas frogs may increase during the winter, reaching a peak in or prior to early April. In the South Dakota animals, the metabolic rates of all three acclimation groups may continue to drop through November. Dunlap (1969) reported very low rates of metabolic activity in both warm and cold acclimated South Dakota *Acris* in early April. This suggests that in winter the frogs maintain low metabolic rates even at moderate temperatures, then, after emerging from hibernation "switch" to a higher metabolic level as found by 16 April in this study.

Precht (1958) points out that the many documented cases of seasonal and latitudinal variation in metabolic rates as well as short-term acclimation in these rates are apparently adaptive. He suggests that their biological significance lies in their enabling the same animal at different times, or populations in different localities, to at least partially stabilize the rates of vital functions largely independent of environmental temperature. In view of the evidence presented by Precht and many others, it seems reasonable to expect that the clearcut seasonally related changes in the rates of oxygen consumption in *Acris* and the consistent differences between the populations of *Acris* are adaptive. Consequently, we shall examine the available information with respect to seasonal temperature trends and the relations between body and ambient temperature in natural populations of *Acris* to see whether they show any consistent relationship to the metabolic data presented in this paper.

One of the striking features of the metabolic rate-time of year curves shown in Figures 1 and 2 is the consistently higher metabolic rates of frogs acclimated at 25° C as compared to those acclimated at the lower temperatures throughout the spring and summer. In the fall of the year the pattern changes with acclimation effects becoming much less pronounced. Consequently, before we can understand what significance these metabolic rate-temperature relationships have for field populations, we need to know when the frogs are most likely to be warm acclimated and when they are likely to be cold acclimated. To gain an insight into this problem we need information (1) on the normal local variations in the temperatures to which the frogs are subjected, (2) on the thermo-regulatory abilities of *Acris* under varying temperature regimes, and (3) on the temperatures to which the frogs acclimate when subjected to daily thermoperiods in body temperature.

Information on the first point is provided in Figures 1 and 2. These figures show, in addition to the metabolic rates, the mean daily maximum and minimum temperatures for Vermillion, South Dakota (Spuhler, Lytle and Moe, 1967) and Austin, Texas (Blood, 1960) for each month for which metabolic data are available.

Fitch (1956) and Brattstrom (1963) have presented data which are pertinent to the second point. They show that the body temperature of *Acris* fluctuates less, both seasonally and daily, than might be expected on the basis of environmental temperatures alone. Fitch reported that, for Kansas *Acris*, over half of the body temperatures of 102 frogs recorded throughout the year were between 28.0 and 31.7° C. On cool sunny days he found that a frog's temperature may exceed air temperature by 10° C or more. In warm weather, they basked less and were usually nearer air temperature. Brattstrom, using his own and Fitch's data, is in general agreement with Fitch and adds information concerning nocturnal thermoregulation. In summary, their data suggest that on sunny days in spring and autumn when maximum air temperatures reach or exceed 15° C, basking temperatures of 25° C or more may be attained. Throughout much of the spring and summer basking temperatures of 25–30° C are common and at night, body temperatures drop to levels that, on the average, lie between ambient air temperature and water temperature.

Information relating to the third point, *i.e.,* the temperature to which the frogs acclimate when subjected to a variable thermal environment, is provided by several investigators. Heath (1963) working on the fish, *Salmo clarki,* and Hutchison and Ferrance (1970) and Seibel (1970) working on the frog, *Rana pipens,* reported that when the animals were subjected to a daily thermo-period, the critical thermal maximum acclimated to the maximum temperature during the period. Consequently, it seems reasonable to expect that the same relationship may apply with respect to acclimation in metabolic rates in *Acris.*

Based on the above information we will assume that *Acris* acclimates to the basking body temperature rather than the lower body temperatures found at night and that in sunny weather whenever air temperatures reach or exceed 15° C, basking temperatures of 25° C or more may be attained. Consequently, as may be seen from the curves for the mean maximum daily temperatures (Figs. 1 and 2), Texas frogs could, on the average, be expected to be warm acclimated throughout the period from early April to October and also during periods of clear, sunny weather later in the season. The South Dakota frogs would usually be warm acclimated from early June to September and, in clear weather, from April into October. If this admittedly simplified analysis is at all accurate then the curves of the warm acclimated frogs are ecologically the most meaningful of the curves throughout much of the study period.

Dunlap (1972) has presented evidence for a zone of metabolic insensitivity to temperature on the acutely measured metabolic rate-temperature (R-T) curves for warm-acclimated *Acris* from Texas and South Dakota. This zone extends between body temperatures of 15–25° C for South Dakota frogs and between 20–30° C for Texas frogs in July. The mean minimum daily temperature for July for each locality lies within the range of temperatures for which metabolic stability can be maintained. Conseqeuntly, it was suggested that this R-T pattern, together with the thermoregulatory ability of *Acris,* reflects an adaptation enabling the frogs to maintain high and constant metabolic rates even in the face of rather wide ranges in environmental temperature between night and day and from day to day. It was further suggested that each population was adapted to the range of temperature, particularly the normal minimal temperature the frogs are exposed to during the daily temperature cycle. This was suggested not only by the shift of the metabolic plateau in South Dakota frogs some 5° C to the left relative to that of the Texas frogs, but also by the fact that the entire warm acclimated R-T curve extending between 5 and 35° C was so shifted. Consequently, Texas frogs which were acclimated and determined at 15° C had uptakes that were lower than those of South Dakota frogs determined at 15° C but similar to those of warm acclimated South Dakota frogs determined at 10° C. Similarly, South Dakota frogs determined at 15° C had uptakes that were similar to those of Texas frogs determined at 20° C.

The data presented in the present paper show that, for warm acclimated frogs, the oxygen uptake at 15° C averages about 50 μl/g per hr greater for the South Dakota frogs than for the Texas frogs from late May to early October. Throughout the bulk of this period, mean minimal daily temperatures lie near or above 20° C on a monthly basis for Austin and near or above 15° C for Vermillion. As seen earlier, the South Dakota frogs at 15° C have metabolic rates similar to those of Texas frogs at 20° C. This, together with the insensitivity of the metabolic rates to temperature over a wide temperature range above 15° C (South Dakota) or above 20° C (Texas), would result in the metabolic rates of the two populations being about the same throughout the late spring and summer in spite of the lower average temperatures to which the South Dakota populations are exposed.

Marked seasonal changes in the metabolic rates of intact frogs of several species of *Rana* (Krogh, 1904; Dolk and Postma, 1927; Stangenberg, 1955; and Fromm and Johnson, 1955) and in *Acris crepitans* (Dunlap, 1969) have been reported. The seasonal changes frequently include a period in the spring when metabolic rates are considerably higher than at other seasons. This period of high oxygen consumption has usually been considered to be related to an increase in activity associated with the spawning season. However, reproductive activity alone is insufficient to account for the early spring peaks in metabolic rates in *Acris*. The peak in breeding activities in both populations occurs after the early spring metabolic peak when metabolic rates have dropped to the summer levels. In South Dakota for example, the metabolic rates are high in late April and early May but have dropped to the summer levels by the end of May. In this area, male *Acris* frequently begin calling by the middle of May, but the major breeding choruses are found in later May and in June (Dunlap, personal observations). In the Texas frogs, high metabolic rates were found in early April but by the end of April the rates had dropped to summer levels. Near Austin, Texas, although chorusing may begin in late January or February, the first records of amplexus were not found until the middle of March and breeding may continue at intervals throughout the summer (Blair, 1961).

An alternative explanation for the early spring metabolic peak in *Acris crepitans* is that it simply represents another example of metabolic compensation for temperature. This results in the maintenance of the metabolic rates of early spring animals at summer levels even though environmental temperatures, and presumably body temperatures, are below summer levels.

As was pointed out earlier (Dunlap, 1972), metabolic rates for warm acclimated frogs at temperatures of 10° C for the South Dakota and 15° C for the Texas frogs are approximately the same for the two populations and average about 50 μl O_2/g per hr less than the stabilized summer rates. These temperatures are also about 5° C below the lower threshold temperatures permitting the stabilized summer rates. If the mean minimal body temperature for active *Acris* in late April and early May in South Dakota and in March and early April in Texas averaged about 10 and 15° C, respectively, then the high rate of oxygen consumption at that time would compensate for the lower temperatures. This is most readily seen from the Texas seasonal curve (Fig. 2). On that curve, the mean rate on 8 April determined at 15° C (180 μl O_2/g per hr) is almost identical to the July rates between 20–30° C (Dunlap, 1972). Although the mean minimal air temperature in April is a little less than 15° C, Brattstrom (1963) found that at night when water temperatures were higher than air temperatures *Acris* would thermoregulate by moving alternately between the water and the shore and consequently body temperatures averaged somewhat above air temperature. The rates of South Dakota frogs determined at 15° C on 16 April and 7 May are even higher than the July rates between 15 and 25° C (Dunlap, 1972). This suggests that the compensation is for a greater than 5° C temperature differential and indeed, the mean minimal temperature for Vermillion in April is well below 10° C. Much more information is needed on the ranges of body temperatures that are characteristic of the frogs in early spring before a more accurate analysis can be made.

A possible model for this switch in the metabolic response to temperature exists in the same apparent mechanism described for the difference in metabolic responses between northern and southern populations (Dunlap, 1972), *i.e.,* a translation of the acute R-T curves to the left in the early spring and back toward the right in summer frogs. Such a change might be mediated by response to photoperiod or perhaps, to a photoperiod-temperature interaction. This interpretation is supported for the Texas frogs by the data shown in Figure 3. Here it can be seen that the Q_{10} for warm acclimated frogs is approximately 1.0 between 15 and 25° C in the early April samples while it is over 2.0 in the later April and in the July samples (Dunlap, 1972). This suggests that the plateau of metabolic insensitivity between 20 and 30° C in the July frogs has been shifted at least 5° C to the left in the early spring frogs.

In October changes occur in the effects of acclimation on the metabolic rates of both Texas and South Dakota frogs. The changes, however, are different for the two populations. During this period the metabolic rates of Texas frogs which are acclimated at 25° C exhibit little change from the summer rates. However, the mean metabolic rates of those acclimated at 15° C and, to a lesser extent, those of frogs acclimated at 5°C, rise to levels approximating those of warm acclimated frogs. In the frogs from South Dakota, there is an increase in the mean metabolic rates of the frogs acclimated at 5 and 15° C in early October followed by a sharp drop in the rates of all acclimation groups to levels approximating those of cold acclimated spring frogs.

As a result of this change in pattern, the Texas frogs can maintain relatively high metabolic rates on warm days in the fall and winter regardless of the temperature to which they have previously been acclimated. This metabolic pattern would seem to be consistent with the activity patterns which have been reported for *Acris* in Texas. Thus, Pyburn (1958) reported that in the vicinity of Austin, Texas, *Acris* is active throughout the year although the number of active frogs decreases in December and January.

The increase in the metabolic rates of cold acclimated South Dakota *Acris* noted in early October may have an adaptive significance similar to that of the pattern noted in the Texas frogs. That is, it would allow the frogs to be active on warm autumn days even though they had been cold acclimated. The abrupt drop in metabolic rates regardless of the acclimation history noted in South Dakota frogs later in the season may well be related to the conservation of energy reserves during the period prior to hibernation and during hibernation. The reduction of metabolic rates in South Dakota *Acris* in the winter is consistent with the 4–5 month period of hibernation in these frogs (Dunlap, 1971).

I wish to thank Mr. Joe Ideker for supplying the Texas frogs used in this study and Mr. Gerald Polcyn for technical assistance. This investigation was initiated under Grant No. GB-5298 from the National Science Foundation and supported in part by the General Research Fund of the University of South Dakota.

Summary

Samples of cricket frogs were collected in South Dakota and Texas at intervals throughout the spring, summer and autumn and acclimated for 5–7 days at 5, 15 and 25° C. Routine metabolic rates were determined at 15° C for acclimated frogs from both localities. Sample of Texas frogs collected in early April, late April and December were determined at 25° C as well.

There were no statistically significant differences between the means of samples acclimated at 5 and 15° C and determined at 15° C on any date for either the South Dakota or the Texas frogs. Except for Texas frogs acclimated at 25 and 5° C in November, no significant differences were found among the means of the three acclimation groups from either locality from October into December. From April through July, with the exception of the Texas samples of 31 May, the frogs from both localities which were acclimated at 25° C had metabolic rates which were consistently higher than and significantly different from frogs acclimated at either 5 or 15° C.

When frogs which had been collected at different times of the year and acclimated at the same temperature were compared, significant seasonal effects were found in all series except the Texas frogs acclimated at 5° C. Several pronounced seasonally related patterns were found. Low metabolic peaks were found in the 5 and 15° C acclimated frogs in late May and early October in both Texas and South Dakota frogs. In the South Dakota frogs acclimated at 25° C, the metabolic rates were highest in April and early May. The rates dropped somewhat in late May and remained fairly uniform until early October. In later October and November the metabolic rates of the warm acclimated frogs from South Dakota reached the lowest levels of the year. In the Texas frogs acclimated at 25° C and determined at 15° C, rates were highest in early April, dropped off by early May and remained fairly uniform into November. There was an indication of a slight increase in the metabolic rates of the warm acclimated frogs in December.

The metabolic rates of Texas and South Dakota frogs with the same acclimation history were compared at approximately the same time of year. In no case were there significant differences attributable to locality for frogs acclimated at either 5 and 15° C. For frogs acclimated at 25° C, the mean metabolic rates of the South Dakota frogs were consistently higher than those of seasonally comparable Texas frogs from April to October and showed statistically significant differences for the 29 April–7 May, July and October comparisons.

For the Texas frogs, the presence of inverse acclimation shown in the April frogs at 15° C with the warm acclimated animals having higher metabolic rates than those acclimated at 5 or 15° C, is replaced by either a weak or no acclimation effect at 25° C.

The results of this study suggest that the pattern of inverse compensation at 15° C reported for *Acris* in earlier papers persists throughout much of the spring and summer in both the South Dakota and the Texas populations. The data further demonstrate the tendency for the metabolic rates of warm acclimated

frogs from South Dakota to be higher at determination temperatures of about 15° C than comparable rates in Texas frogs. The seasonally related differences in the metabolic rates and also the differences in the metabolic rates of frogs from the two localities are interpreted in terms of published data on body temperature-environmental temperature relationships in *Acris* and with published climatological data. The data are considered to be consistent with the hypothesis of the role of metabolic patterns in the maintenance of metabolic stability in thermally instable environments.

LITERATURE CITED

BLAIR, W. F., 1961. Calling and spawning seasons in a mixed population of anurans. *Ecology,* **42**: 99-110.

BLOOD, R. D. W., 1960. Climates of the states; Texas. No. 60-41, U.S. Dept. Commerce Weather Bureau, *Climatography of the United States.* U.S. Government Printing Office, Washington, 28 pp.

BRATTSTROM, B. H., 1963. A preliminary review of the thermal requirements of amphibians. *Ecology,* **44**: 238-255.

BULLOCK, T. H., 1955. Compensation for temperature in the metabolism and activity of poikilotherms. *Biol. Rev.,* **30**: 311-342.

DOLK, H. E., AND N. POSTMA, 1927. Über die Haut und die Lungenatmung von *Rana temporaria. Z. Vergl. Physiol.,* **5**: 417-444.

DUNLAP, D. G., 1969. Influence of temperature and duration of acclimation, time of day, sex and body weight on metabolic rates in the hylid frog, *Acris crepitans. Comp. Biochem. Physiol.,* **31**: 555-570.

DUNLAP, D. G., 1971. Acutely measured metabolic rate-temperature curves in the cricket frog, *Acris crepitans. Comp. Biochem. Physiol.,* **38A**: 1-16.

DUNLAP, D. G., 1972. Latitudinal effects on metabolic rates in the cricket frog, *Acris crepitans:* Acutely measured rates in summer frogs. *Biol. Bull.,* 143: 332-343.

FITCH, H. S., 1956. Temperature responses in free-living amphibians and reptiles of northeastern Kansas. *Univ. Kansas Publ. Mus. Natur. Hist.,* **8**: 417-476.

FROMM, P. O., AND R. E. JOHNSON, 1955. The respiratory metabolism of frogs as related to season. *J. Cell. Comp. Physiol.,* **45**: 343-359.

FRY, F. E. J., 1957. The aquatic respiration of fish. Pages 1-63 in M. E. Brown, Ed., *The Physiology of Fishes,* Vol. 1. Academic Press, New York.

HEATH, W. G., 1963. Thermoperiodism in sea-run cutthroat trout (*Salmo clarki clarki*). *Science,* **142**: 486-488.

HUTCHISON, V. H., AND M. R. FERRANCE, 1970. Thermal tolerances of *Rana pipiens* acclimated to daily temperature cycles. *Herpetologica,* **26**: 1-8.

HUTCHISON, V. H., W. G. WHITFORD AND M. KOHL, 1968. Relation of body size and surface area to gas exchange in anurans. *Physiol. Zool.,* **41**: 65-85.

JAMESON, D. L., W. TAYLOR AND J. MOUNTJOY, 1970. Metabolic and morphological adaptation to heterogenous environments by the Pacific tree toad, *Hyla regilla. Evolution,* **24**: 75-89.

LI, J. C. R., 1957. *Introduction to Statistical Inference.* Edwards Brothers, Ann Arbor, 568 pp.

KROGH, A., 1904. On the cutaneous and pulmonary respiration of the frog. (A contribution to the theory of the gas exchange between the blood and the atmosphere.) *Skand. Arch. Physiol.,* **15**: 328-419.

OSTLE, B., 1963. *Statistics in Research.* (2nd. Ed.) Iowa State University Press, Ames, 585 pp.

PRECHT, H., 1958. Concepts of the temperature adaptation of unchanging reaction systems of cold-blooded animals. Pages 50-77 in C. L. Prosser, Ed., *Physiological Adaptations.* American Physiological Society, Washington.

PROSSER, C. L., 1955. Physiological variation in animals. *Biol. Rev.,* **30**: 229-262.

PROSSER, C. L., 1958. General summary: The nature of physiological adaptation. Pages 167–180 in C. L. Prosser, Ed., *Physiological Adaptations,* American Physiological Society, Washington.

PYBURN, W. F., 1958. Size and movements of a local population of cricket frogs (*Acris crepitans*). *Texas J. Sci.,* **10**: 325–342.

SEIBEL, R. V., 1970 Variables affecting the critical thermal maximum of the leopard frog, *Rana pipiens* Schreber. *Herpetologica,* **26**: 208–213.

SPUHLER, W., W. F. LYTLE AND D. MOE, 1967. *Climatological Summary No. 8; Vermillion, South Dakota.* Agricultural Experiment Station, South Dakota State University, Brookings, 6 pp.

STEBBINS, R. C., 1951. *Amphibians of Western North America.* University of California Press, Berkeley and Los Angeles, 539 pp.

STANGENBERG, G., 1955. Der Temperatureinfluss auf Lebensprozesse und den Cytochrom c-Gehalt beim Wasserfrosch. *Pfluegers Arch. Gesamte Physiol. Menchen Tiere,* **260**: 320–332.

TASHIAN, R. E., AND C. RAY, 1957. The relation of oxygen consumption to temperature in some tropical, temperate and boreal anuran amphibians. *Zoologica,* **42**: 63–68.

VERNBERG, F. J., 1962. Comparative physiology: Latitudinal effects on physiological properties of animal populations. *Annu. Rev. Physiol.,* **24**: 517–546.

Reference: *Biol. Bull.*, **145**: 119–126. (August, 1973)

GROWTH RATE, LONGEVITY AND MAXIMUM SIZE OF *MACOMA BALTHICA* (L.)

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Macoma balthica (Linnaeus, 1758) has long been recognized as a dominant species in marine bottom communities ringing the boreal North Atlantic Sea and in the Arctic Seas (Thorson, 1957). Coan (1971) has recently synonymized *Tellina inconspicua* Broderip and Sowerby with *M. balthica* extending the range of this taxon into the North Pacific as far south as San Francisco Bay. Here the species is less dominant due probably to the very large number of other *Macoma* species in this region. Such an unusually extensive geographic range affords a rare opportunity to study the way one species adapts to widely varying climatic conditions.

A wealth of material on fundamental aspects of the life history of *M. balthica* has appeared over the past decade correcting a previous paucity of information. Two of these aspects, its growth rate and longevity, have been found to vary widely. Most workers (Vogel, 1959; Segerstrale, 1960; Lavoie, Tremblay and Filteau, 1968; Lammens, 1967; Semenova, 1970; R. H. Green, in preparation) have used annual rings produced by a cessation of growth during the winter to determine age and growth rate. In populations from Massachusetts to Chesapeake Bay, however, these bivalves do not display these rings (McErlean, personal communication). Neither can the difference in successive modes in size-frequency distributions be used to age these animals, because there are usually only two peaks—young of the year and adults. Since rates vary widely, I studied a population of this species at Rand Harbor, Falmouth, Massachusetts to determine growth rate and longevity in a southern population.

METHODS

Sixty specimens of *M. balthica* ranging from 6 to 25 mm in length were measured to the nearest 0.1 mm using vernier calipers and tagged with numbered squares of adhesive tape glued to the left valve with a drop of fast-drying jeweler's cement. The India-ink numbers were protected by a coat of clear nail polish. The bivalves were then released (March 16, 1970) into a large wooden box (38 × 58 × 13 cm) filled with native sediment and placed flush with the sediment surface at one foot below mean low water (MLW) in Rand Harbor. The box was removed seasonally (May 21, August 13, November 27, 1970 and March 31, 1971), and the tagged animals were carefully screened from the sediment, measured and replaced within 12 hours.

Since variation in biomass/m^2 at a particular site may cause differences in growth rate, both the natural density and size distribution of *M. balthica* were examined at Rand Harbor. In October 1969 nine samples 62.5 cm^2 in area were taken to 15 cm depth at each of four stations along a transect extending from the salt marsh to one foot below MLW. All specimens of *M. balthica* were sieved from the sediment, counted and measured. Here as elsewhere (Segerstrale, 1960), the size distribution was found to vary with water depth, making improbable the existence of a "usual" size distribution. Therefore animals for this study were selected so that all lengths found at Rand Harbor in March 1970 would be well represented. Densities ranged from 184/m^2 at one foot below MLW to 492/m^2 at MLW in October 1969 so the experimental density of 272/m^2 was well within the natural range for this site.

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Macoma balthica burrows deeper as it grows (Lammens, 1967; Gilbert, 1969); spat live in the top 1 mm while the largest adults can live up to 18 cm deep. Since the box was not this deep, a controlled experiment was run in July 1970 to determine if this or other factors affected growth. Two sets of 12 bivalves of equal length ($\bar{x} \pm 1$ s.d. $= 11.7 \pm 2.1$ mm; 11.9 ± 2.1 mm; $t_{22} = 0.233$) were tagged as above. One set was released in a small box (38 × 28 × 13 cm) in the manner described above and the second in a comparable area of undisturbed sediment marked off by a bottomless frame (38 × 28 cm). It was hoped that control animals would resume their normal living position, yet not move beyond the confines of the frame. Sizes were small and density low so that optimal growth could be observed. Three weeks later the animals were recovered and measured as above.

The dry-weight biomass and dry shell weight of 14 animals collected at Rand Harbor on May 11, 1970 and 23 collected at Sagadahoc Bay, Georgetown, Maine, on January 1, 1972 were determined by carefully removing all flesh from the shell and drying tissues and shell separately for 24 hours at 70° C. The animals from Rand Harbor were separated on the basis of gonadal development; eight had ripe gonads and six had recently spawned. Those from Sagadahoc Bay had very small gonads if present. The caloric content per ash-free gram of dry-weight biomass was determined for eight animals from Rand Harbor (four collected on May 11 and four on August 28, 1970) using methods and a calorimeter described by Phillipson (1964).

RESULTS

In the controlled experiment recovery was low; nine from the box and six from under the open frame. This is probably due to two factors: First, evidence of green crab and *Urosalpinx cinerea* predation, crushed and bored shells in the presence of both species, was abundant during this month and, secondly, several bivalves were probably missed due to the difficulty of removing all the sediment from the area under the open frame to a depth of 15 cm. The mean initial lengths of the recovered bivalves were still not significantly different ($\bar{x} \pm 1$ s.d. $= 11.05 \pm 0.48$ mm; 11.69 ± 0.45 mm; $t_{13} = 0.179$). The mean growth was not significantly affected by enclosure but the animals in the open frame grew somewhat more (open frame $\bar{x} \pm 1$ s.d. $= 3.7 \pm 0.79$ mm; box $\bar{x} \pm 1$ s.d. $= 2.4 \pm 0.15$ mm: $F_{1,13} = 2.54$, n.s.).

Mortality in the year-round study was 15% ($n = 9$) and varied little by recapture date (Fig. 1, insert). There was no emigration or loss due to predation,

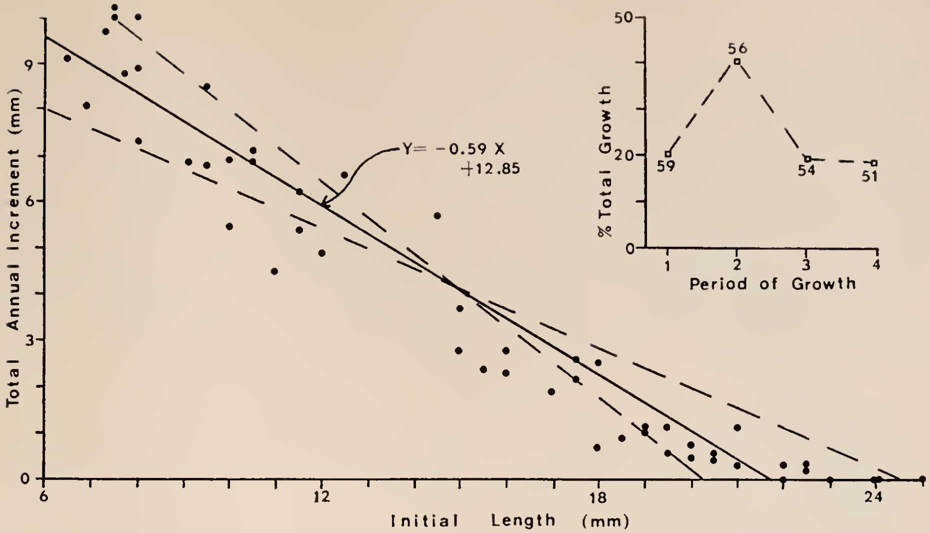

FIGURE 1. Total annual increase in length of 51 specimens of *M. balthica* of varying initial lengths. Dotted lines indicate 95% confidence intervals for the slope. Insert: seasonal growth as a percentage of the total annual growth; sample sizes are indicated for each period of growth (1 = 3/16–5/21/70; 2 = 5/21–8/31/70; 3 = 8/13–11/27/70; 4 = 11/27/70–3/31/71).

although the bivalves in the neighboring experiment suffered heavy losses. *M. balthica* living throughout the year (n = 51) was used in the calculations below. Although the original number of marked bivalves is low for the mark-and-recapture method, the ability to recapture every individual at each sampling period resulted in data comparable to that resulting from studies using free-ranging animals (*e.g.* Frank, 1969).

Growth occurs steadily throughout the year except during the summer when it accelerates sharply (Fig. 1). The summer growth period starts around May, when this population spawns and evidently ends abruptly at the beginning of August, when gonadal regeneration begins (Gilbert, unpublished data). The relationship between initial length (X) and total annual increment (Y) can be expressed by the equation $Y = -0.59\ X + 12.85$ with a 95% confidence interval for the slope of −0.64 to −0.54 (Fig. 1).

The growth of the 1970 year class in the present study was followed by measuring the small, unmarked specimens of *M. balthica* first found in the box in August where they probably settled as spat. The wide size range (3.4–7.4 mm) of these juveniles in August suggests that spatfall occurs from May into the summer. They were returned to be censused again in November when they had attained sizes from 7.0–12.7 mm ($\bar{x} = 9.66$ mm; s.d. = 1.61; n = 22).

A growth curve (Fig. 2) was constructed for the population at Rand Harbor assuming that the observed rates apply to the whole population and that the climate in 1970–71 was typical. Surface sea water temperature data taken at the Buzzards Bay light station show that 1970 was an average year; the mean 10-day average for 1970 was 10.56° C while the overall average for the years 1956–70

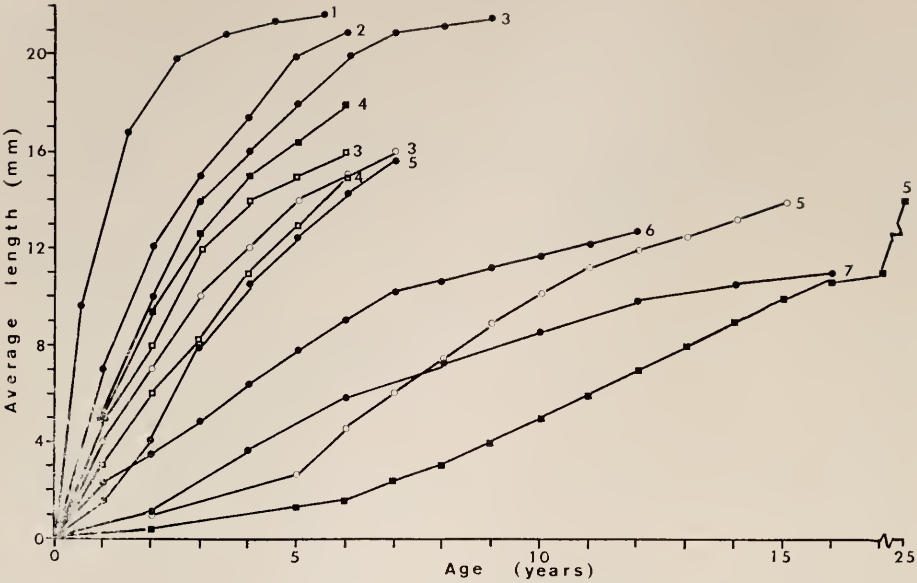

FIGURE 2. Growth curves of populations of *M. balthica* in different localities. The locations are: (1) Rand Harbor (−0.3 m); (2.) Wadden Zee, The Netherlands; (3.) (Vogel) North Sea (filled circle), Kiel Bay (open square), Gulf of Finland (6 m; open circle) (4.) Hudson's Bay, 1.1 m (filled square), MLW (open square); (5.) Gulf of Finland (Segerstrale), 3 m (filled circle), 20 m (open circle), 35 m (filled square); (6.) St. Lawrence River; (7.) Kandalski Bay, White Sea. For further details see Table I.

was 10.27° C (Chase, 1972). The curve was constructed in segments: the growth for the first $\frac{1}{2}$ year is the average growth of the spat found in the box in August and measured again in November; later segments were constructed using the relationships in Figure 1. The resulting curve shows that at Rand Harbor, *M. balthica* attains most of its adult length within the first two years of its life. Then growth tapers and finally ceases for an unknown period once a size of 22+ mm has been reached. This study and others' findings suggest a total life span of 6–10 years in Rand Harbor.

There were no significant differences in the following relationships: $\log_{10}$ dry weight biomass *versus* $\log_{10}$ shell length in animals with ripe *versus* those with spawned gonads at Rand Harbor; $\log_{10}$ shell weight *versus* $\log_{10}$ shell length and $\log_{10}$ dry weight biomass versus $\log_{10}$ shell length in Maine *versus* those relationships in Massachusetts. The combined regression of dry shell weight (Y) on shell length (X) was $\log_{10} Y = 2.98 \log_{10} S - 0.23$ and that of dry weight biomass (Y′) on shell length (X) was $\log_{10} Y' = 3.05 \log_{10} X - 1.12$. The combined regression coefficients (2.98; 3.05) were not significantly different indicating that these two dimensions show a similar allometric growth with a constant of allometry of approximately 3 (Simpson, Roe and Lewontin, 1960). There was no significant difference between the mean caloric content of animals collected in May and August; the average of the eight replicates was 4.049 kcal/ash-free g. All statistics were calculated according to Sokal and Rohlf (1960).

DISCUSSION

In *M. balthica* data presented here suggest that maximum size and growth rate decreases and longevity increases with increasing latitude and relatedly cooler temperatures and shorter growing seasons (Table I and Fig. 2). Nutritive conditions and changes in salinity have formed the basis of earlier theories accounting for the variability in the maximal size. Segerstrale (1960), finding that both size and temperature decreased with depth (Sta. I-XLIV), concluded that, since the magnitude of temperature change was not sufficient to explain the decrease in size, growth was stunted because the available food becomes nutritively poorer in deeper waters (page 62). Segerstrale's temperature data, however, indicate a considerable lag in the warming of deeper waters and a progressively shortened

TABLE I

Comparison of temperature, longevity, and maximum size of Macoma balthica *at various locations*

Locality	°N. lat.	Average longevity (years)	Max. size (mm)	Water temperature (°C)	Source
Patuxent River	38–39				
Sta. 4			31–32	19 (Average spring temperature, Potomac River)	McErlean, 1964; Pfitzenmeyer and Drobeck, 1963
6			25–26	17	
8			27–28	14	
10			27–28	10	
Rand Harbor, Buzzards Bay, Massachusetts	41 30′	6–10	26	14 (May 1970)	Gilbert
Wadden Zee, Den Helder, Netherlands	53	6	23	14 (May 1963)	Lammens, 1967
Gulf of Finland, Tvarminne, Finland	60		(mean max.)		Segerstrale, 1960
Sta. I (3m)		7–8	20.9	6–11 (May 1927–32)	
Sta. XXVI (20 m)			18.4	1–5 (May 1929–32)	
Sta. XLIV (35 m)			17.3	1–4 (May 1929–32)	
North Sea		9	22		Vogel, 1949
List, Sylt, and	55				
Norderney, Germany	54				
Kiel Bay, Germany	54 30′	6	15		Vogel, 1949
Gulf of Finland, Tvarminne, Finland (6 m)	60	7	15		Vogel, 1949
Kandalski Bay, White Sea, USSR	66	25–30	17	7 (May 1962)	Semenova, 1970
St. Lawrence River					
Cacouna, Quebec	48	12	13	4 (May 1967)	Lavoie *et al.*, 1968
Hudson's Bay Churchill, Manitoba	58 46′				Green, in preparation
1.1 m		8	17	−5 to 10 (Average May)	
MLW		?	13	9 (July 5, 1970)	
Aberlady Bay, Firth of Forth, Scotland	56	2	8–9	12 (Mean air temp. May to Sept.)	Stephen, 1931 and 1938

growing season for benthic fauna. Vogel (1959) found decreasing size with decreasing salinity in a series of stations from the North Sea into the Baltic Sea, while McErlean (1964) also found a decrease in size, but with an increase in salinity in the Patuxent River estuary in Chesapeake Bay; neither author presented temperature data. Pfitzenmeyer and Drobeck (1963), however, present temperature and salinity data for stations in the Potomac River, adjacent to the Patuxent. When stations of similar salinities in these two rivers are compared, it is evident that both temperature and maximum size increase as salinity decreases. In any case, it is very unlikely that salinity had an important effect, considering the very wide salinity tolerance of this species (Lavoie, 1970; Bagge, 1965). Thus both formerly conflicting data and variable maximal sizes in this species can be most simply explained as a function of the hydroclimate: with warmer temperatures and a longer growing season, a larger size is attained. The one exception seems to involve a transient population in Scotland (Stephen, 1931) that barely reaches a size that is reproductive at Rand Harber. Hydrographic conditions and selective predation have been shown to remove size classes shortening the life span at other sites (Lavoie, 1970; Semenova, 1970). Such factors may be acting on the Scottish population.

Growth rate and longevity are also related to temperature: at higher temperatures *M. balthica* grows faster, but seems to have a shorter life span (see also Segerstrale, 1960; R. H. Green, in preparation). The influence of temperature on growth rate and maximum size is evident in Green's study (number 4 on Fig. 2). Although food availability is similar, specimens of *M. balthica* at 1.1 m above MLW grew faster and reached larger sizes than those at MLW; the former are exposed about 37% of the time to 15–18° C air temperature during July and August while the latter are usually covered by cold arctic water. A similar pattern is found in intertidal populations of *Mytilus californianus* where the growth rate of an Alaskan population is lower than Californian ones (Dehnel, 1956). Studies on temperature compensation in other molluscs have shown that measures of metabolic rate, such as rate of heart beat and oxygen consumption, are higher in cold-adapted animals, *i.e.* those living at the northern end of a species range or at the lower end of the intertidal (Segal, 1956, 1961). Cold-adapted *M. balthica* may therefore be expending more energy on respiration and less on growth than warm-adapted individuals.

At Rand Harbor, most *M. balthica* larger than 22 mm were not observed to grow during the study year (Fig. 1). Elsewhere they continue to grow throughout the life span but so slowly that 22 mm is never reached despite life spans up to 25 years (Fig. 2). Although a smaller ultimate size may be intrinsic at more northernly sites, the shape of the growth curves disputes this conclusion. Although the inverse relationship between longevity and growth rate is well documented, the reason why it occurs is still unknown.

Macoma balthica and *Scrobicularia plana*, a larger, related, also deposit-feeding species that often replaces *M. balthica* in more saline flats (Spooner and Moore, 1940), exhibit the same allometry of size. Hughes (1970) found an average regression coefficient of 3.00 for the relationship of $\log_{10}$ dry-flesh weight (g) to $\log_{10}$ shell length (mm) and 3.41 ± 0.82 for $\log_{10}$ shell weight to $\log_{10}$ shell length. *Scrobicularia plana* also has a low caloric content (5.197 kcal/ash-free g: Hughes, 1970). Slobodkin (1962) considered *Ensis minor* with a caloric content of 3.5 kcal/ash-free g to be an exception to other animals examined which fell in the higher range of 5:5–7.5 kcal/ash-free g. The evidence seems to be mounting, however, that bivalve mollusks as a group have a low caloric content perhaps due to their low lipid content (Hughes, 1970).

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SUMMARY

1. Growth rate and longevity were determined for a population of *M. balthica* at Rand Harbor, Falmouth, Mass., using a modification of the mark and recapture technique that allowed all marked individuals to be followed throughout a year. Dry weight biomass, dry shell weight and caloric content were also determined.

2. Caloric content is 4.049 kcal/ash-free g. Dry weight biomass and dry shell weight were found to have a similar allometric growth with a constant of allometry of approximately 3 when measured against shell length.

3. The bivalves were discovered to grow year round with the peak period of growth occurring from May to August. A negatively linear relationship between initial length (X) and total annual increment (Y) was found ($Y = -0.59\ X + 12.85$).

4. The growth curve for the Rand Harbor population revealed that within the first two years of life, *M. balthica* attains most of its total length. Growth ceases once a maximum size of 22–25 mm is reached and total longevity is estimated to be 6–10 years.

5. These results were compared to other studies and the theory that maximum size, growth rate and longevity are controlled by temperature presented and discussed.

LITERATURE CITED

BAGGE, P., 1969. Effects of pollution on estuarine ecosystems. *Merentutkimuslaitoksen Julkaisu Havsforskningsinst. Skr.*, 228: 3–130.

CHASE, J., 1972. Oceanographic observations along the east coast of the United States:, January–December 1970. *U. S. Coast Guard Oceanogr. Rep.*, no. **53**: 7–145.

DEHNEL, P. A., 1956. Growth rates in latitudinally and vertically separated populations of *Mytilus califorianus*. *Biol. Bull.*, **110**: 43–53.

COAN, E. V., 1971. The Northwest American Tellinidae. *Veliger*, **14** (Supplement): 1–50.

FRANK, P. W., 1969. Growth rates and longevity of some gastropod mollusks on the coral reef at Heron Island. *Oecologia*, **2**: 232–250.

Gilbert, M. A., 1969. Interspecific relationships between *Mya arenaria, Macoma balthica,* and *Gemma gemma* as revealed by distribution patterns. *M.S. special problem, University of Massachusetts, Amherst,* 17 pp.

Hughes, R. H., 1970. An energy budget for a tidal-flat population of the bivalve *Scrobicularia plana* (Da Costa). *J. Anim. Ecol.* **39**: 357–381.

Lammens, J. J., 1967. Growth and reproduction in a tidal population of *Macoma balthica* (L.). *Netherlands J. of Sea Res.,* **3**: 315–382.

Lavoie, R., 1970. Contribution a la biologie et a l'ecologie de *Macoma balthica* (L.) de l'estuaire du Saint-Laurent. *Ph.D. thesis, Universite Laval,* 249 pp.

Lavoie, R., J.-L. Tremblay and G. Filteau, 1968. Age et croissance de *Macoma balthica* (L.) a Cacouna-est dans l'estuaire du St. Laurent. *Naturaliste Can.,* **95**: 887–895.

McErlean, A. J., 1964. Characteristics of *Macoma balthica* populations in the Middle Patuxent estuary. *Chesapeake Sci.,* **5**: 200–208.

Pfitzenmeyer, H. T., and K. G. Drobeck, 1963. Benthic survey for populations of soft-shelled clams, *Mya arenaria,* in the lower Potomac River, Maryland. *Chesapeake Sci.,* **4**: 67–74.

Phillipson, J., 1964. A miniature bomb calorimeter for small biological samples. *Oikos,* **15**: 130–139.

Segal, E., 1956. Microgeographic variation as thermal acclimation in an intertidal mollusc. *Biol. Bull.,* **111**: 129–152.

Segal, E., 1961. Acclimation in molluscs. *Amer. Zool.,* **1**: 235–244.

Segerstrale, S. G., 1960. Investigations on Baltic populations of the bivalve *Macoma balthica* (L.). Part I. *Soc. Sci. Fenn. Comm. Biol.,* **32**: 1–72.

Semenova, N. L., 1970. Lineinyi rost *Macoma balthica* (L.) Kandalakshskon zalive Belogo morya (Linear growth in *Macoma balthica* in the Gulf of Kandalaksha in the White Sea). *Trudy Belomorskoi Biologicheskoi Stantsii Moskovskogo Gosudarstuennogo Universiteta,* **3**: 69–80.

Simpson, G. G., A. Roe and R. C. Lewontin, 1960. *Quantitative Zoology.* (Revised Edition) Harcourt, Brace and Company, New York, 440 pp.

Slobodkin, L. B., 1962. Energy in animal ecology. *Advance in Ecological Research,* **1**: 69–101.

Sokal, R. R., and F. J. Rohlf, 1969. *Biometry.* W. H. Freeman and Company, San Francisco, 776 pp.

Spooner, G. M., and H. B. Moore, 1940. The ecology of the Tamar estuary. IV. An account of the macrofauna of the intertidal muds. *J. Mar. Biol. Ass. U.K.,* **24**: 283–330.

Stephen, A. C., 1931. Notes on the biology of certain lamellibranchs on the Scottish coast. *J. Mar. Biol. Ass. U.K.,* **17**: 277–300.

Stephen, A. C., 1938. Temperature and the incidence of certain species in western European waters in 1932–1934. *J. Anim. Ecol.,* **7**: 125–129.

Thorson, G., 1957. Bottom communities. *Geol. Soc. Amer. Mem.,* **67**: 461–534.

Vogel, K. 1959, Wachstumsunterbrechungen bei Lamellibranchiaten und Brachiopoden. *Neues Jahrbuch fur Geologie und Palaeontologie, Abhandlung B,* **109**: 109–129.

Reference: *Biol. Bull.*, **145**: 127–139. (August, 1973)

BLOOD-FEEDING BEHAVIOR OF ADULT *AEDES AEGYPTI* MOSQUITOES[1]

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Of all the complex actions which mosquitoes perform in their lives, none are of such great interest to human beings as the blood-feeding behavior of the adult females. Except for the facts that the males do not pierce the skin to take blood and that the females readily do so, some of the simplest and most interesting features of blood-feeding have never been described or adequately studied, in spite of the fact that this act has been repeatedly observd since the first description by Reaumur (1738). The most detailed information on blood feeding in mosquitoes is given in Christophers' (1960) review. No one investigator has examined the general problem of blood-feeding behavior of any one species of mosquito so that our current knowledge is based on a series of isolated observations by many workers using different species. Since the work of Robinson (1939) on *Anopheles,* apparently no one has studied the effects of experimental surgery on the mechanism of fascicular insertion by mosquitoes. The present paper offers an overall picture of many aspects of blood feeding by known age individuals of a single species.

METHOODS AND RESULTS

Bangkok and U. S. Naval Medical (U.S.N.M.) strains of the Yellow Fever mosquito, *Aedes* (*Stegomyia*) *aegypti* (Linnaeus), were reared in an insectary held at 27° C and 80% relative humidity. The adults were aged by noting the time when they emerged from the pupal case. The degree of engorgement on fluid was estimated qualitatively using the visual scale of Pilitt and Jones (1972). Unless specified differently, 10 individuals of each sex were employed in each test using a 1 cu ft cage.

Definitions

Since adult mosquitoes feed only on substances which can be drawn into their alimentary canals by suction, the term *feeding* will be defined here to include the uptake of any fluid into the alimentary system via the food canal, the latter being formed within an intricate arrangement of various fine feeding stylets (the fascicle) which are ensheathed by a large labium. The fascicle and the labium together comprise the conspicuous proboscis, at the anterior end of which are two sensory-haired labella.

The term *probing* has been used very loosely with regard to mosquitoes to refer to three very different acts: (1) a directional thrust of the whole proboscis

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without necessarily contacting a specific object, (2) the touching of the labella to the surface of an object, and (3) the insertion of a distal portion of either the fascicle or the proboscis into the test object. The following terms were devised to refer to each of these three kinds of probes: (1) *directional (proboscidal) thrust,* (2) *labellate* or *labellation,* and (3) *fascicular insertion* (where the fascicle alone is inserted) or *proboscidal insertion* (never seen during normal blood feeding).

Reactions of adult male mosquitoes to a human forearm

Exposing a bare human forearm within a 1 cu ft cage containing many resting adult male mosquitoes caused them to fly about almost at once. They made rapid flights along highly variable, usually widely elliptical, paths and often flew in figure-8 patterns. Some males would skim along about 3 inches or less from the arm and return frequently, generally parallel to it. Most of them landed on the hairs and skin. None of the males made any attempt to insert his proboscis or fascicle into the skin. When the tip of the labella did touch the skin, the maxillary palps were often raised. Individuals were observed to return repeatedly to the human forearm with or without labellating for periods up to 2½ hrs, at which time the males appeared noticeably less agitated by the presence of the arm.

Open pool feeding on defibrinated sheep blood or a mixture of blood and sucrose

Males and females which had been fasted for 4 days were placed in a cage with a crucible containing whole sheep blood. While they did not all take blood, those which did so fed to a stage 3 or 4 on the engorgement scale. When the mosquitoes were offered a 1:1 mixture of sheep blood and sucrose, most of them rapidly visited the mixture and imbibed to a stage 4 on the engorgement scale. When males were offered a choice between 10% sucrose and defibrinated sheep blood, they quickly drank only the sucrose.

Initial reactions of adult female mosquitoes to human beings

Female mosquitoes were observed to approach clothed human beings in the laboratory. They almost never took a direct path when the distance between them and the human being was more than 3 ft. They often flew at a level below the waist in a very complex zigzagging path. Once within a range of 12 inches, they tended to fly in a rapid horizontal figure-8 pattern which would alternate with sudden up and down flights. They would rapidly retreat only to approach again. Most of the females landed on the ankles and legs and repeatedly thrust and labellated. Females also landed and fed on different regions of the head and neck.

Females often thrust their proboscides vigorously and repeatedly at a variable but rapid rate through clean mosquito netting to various extents. Such probing responses could generally be rapidly elicited by bringing the cage near a human arm or after breathing lightly on the netting. Females thus activated appeared to go into a probing frenzy, that is, they would make strong directional thrusts, labellate, and in some cases even attempt to pierce various portions of the cage in the immediate vicinity of a human being.

To determine the age at which females would take their first blood meal, 25 unfed mosquitoes of precisely known ages were exposed for 30 min periods to a bare human forearm daily for 4 to 5 hrs per day, from the time of their emergence from the pupal case until they were 4 days old.

When a hand was placed immediately in front of a very young female, no responses were elicited. The earliest that a female of the Bangkok strain was observed to take a blood meal was 21 hrs postemergence. The majority took their first blood meal when they were 23 to 24 hrs old. Some of the U.S.N.M. females were first observed to take a meal when they were 26 hrs old. In one test 3 out of 15 U.S.N.M. females never took a blood meal and died on the fourth day of the study.

Placing a human forearm in a cage of females which are ready to take their first blood meal, agitates some of them to fly. If they are less than 6 to 9 inches from the arm, they tend to land on it very quickly on any particular site. Typically, females land with 4 legs on the skin and either begin walking while making short directional thrusts or light labellar taps before settling on one site for puncture, or they begin almost at once to insert the fascicle at the initial landing site. Females will feed when their dorsal side is either up or down or faces laterally. While mosquitoes characteristically do not stand or feed with their heads facing downwards under normal conditions, it was discovered that if they landed on the arm and it was turned so that the mosquito's head and proboscis faced straight downwards, they could labellate, penetrate and fully engorge on blood. (Upon removing their fascicles, however, most of the mosquitoes somersaulted downwards due to the weight of the blood in their engorged abdomens, at which moment they took flight.)

Once a feeding site is determined, the females always lower their metathoracic legs before starting to puncture the skin. Shortly after the labella are firmly appressed, the maxillary palps are suddenly lifted to an angle of about 75°. Although one palp can be lifted with the other only partially raised, usually they are elevated simultaneously or nearly so. As soon as the fascicle begins to penetrate the skin, the palps begin to move up and down rapidly over a small angle of approximately 15°, and the head and antennae are seen to rock but at a slower rate than the palps. Often after a puncture has been made, a female will run the fascicle up and down within the wound. During puncture, a female's legs usually move slowly closer to her body, as the femoro-tibial angles becomes more acute. As penetration starts, the proximal portion of the labial part of the proboscis curves posteriorly in a hairpin fold, and the fascicle is then desheathed from the labium except at the labella. During penetration, the labium as a whole makes fine side-to-side movements. The fascicle is usually inserted into the skin for about one-half its length, although it may sometimes be pushed down close to the level of the distal end of the maxillary palps. The angle of the proboscis is 45° to 90° to the plane of the skin utilized. Rapid movements of the palps occurred until a bright red streak of blood suddenly appeared in the fascicle. As soon as this was seen, the palps abruptly stopped rocking. The sudden cessation of palpal movements indicates that imbibition has begun: the animal then becomes almost motionless, except for a small, intermittent, telescopic extension and retraction of the tip of the abdomen. When the female reaches a stage 5- on the engorgement scale, she expels the first droplet from her anus. Between stages 5- and 5 she continues to expel droplets. The first 1 or 2 drops

TABLE I

Duration of different phases of blood-feeding of the Bangkok strain of unrestrained Aedes aegypti *females*

Phase of blood feeding	Duration in seconds of different phases of feeding in			
	1 cu. ft. cage*		2 in. plastic cup**	
	Range	Mean and S. E.	Range	Mean and S. E.
Insertion of fascicle	16–105	53.3 ± 6.6	18–120	43.4 ± 5.1
Full engorgement (to stage 5)	103–225	155.3 ± 8.8	45–132	128.9 ± 8.4
Withdrawal of fascicle	2–4	5.4 ± 0.9	2–35	10.9 ± 1.7

* 14 mosquitoes which were 2 days old and fasted.
** 20 mosquitoes which were $3\frac{1}{2}$ days old and fasted.

appear either milky or clear, while subsequent ones are nearly always clear and of variable volumes. Engorged females after leaving the host were observed to expel droplets of clear fluid from the anus at a decreasing rate for a period up to 2 hrs or longer.

Withdrawal of the fascicle at the end of normal blood feeding was indicated by a renewed rocking movements of the maxillary palps, and these continued until the fascicle was fully removed. Fascicular withdrawal was either a simple direct steady removal or consisted of first withdrawing the fascicle and then reinserting it momentarily before total withdrawal. Some females (5%) were seen to reinsert their fascicles after a stage 5 engorgement had been attained and they continued to do this for as long as 1 min, occasionally making a series of reinsertions. During fascicular removal, a female straightens her prothoracic legs to lift her head and thorax, and then makes a backward angling movement with her whole body. During the act of fascicular withdrawal, the labium rocks from side-to-side as the distal end is slowly slid down the emerging fascicle. Once the fascicle has been removed from the skin, it springs upward and forward. Complete re-sheathing of the fascicle occurs after its complete withdrawal from the host. The final stages of re-sheathing consist of a short rapid back-and-forth pumping action of the labium as the fascicle is fitted back within its groove. Occasionally a female augments the final stages of re-sheathing by placing her front tibial spines in the area of the labial groove and slides them forward.

Duration of the three phases of the blood-feeding act

The duration of each of the three phases of the blood-feeding act (insertion of the fascicle, imbibition of blood, and withdrawal of the fascicle), as shown in Table I, was found to be highly variable, despite the uniformity of individuals and test conditions. When a forearm was offered to females held in small containers or in 1 cu ft cages, those in the small cages (2 inch plastic cups) required less time for insertion and engorgement. It can be seen for both groups that the insertion of the fascicle required much longer than for its withdrawal, while the duration of actual blood-intake was longer than other phases.

TABLE II

First and second blood meals of the U. S. Naval Medical strain of Aedes aegypti *females*

Individual number	Hours since first meal	Seconds			
		To locate arm and labellate		From fascicular insertion to withdrawal	
		first meal	second meal	first meal	second meal
1	2	82.0	46.0	176.0	201.0
2	2	7.9	35.7	202.9	158.7
3	2	44.4	28.6	315.0	205.0
4	3	89.6	32.4	374.6	165.7
5	5	58.9	32.8	352.5	130.3
Mean	3.0	56.6	35.1	284.2	172.1
S.E.	±0.6	±16.3	±3.5	±39.7	±14.9

The second blood meal

Immediately after taking a full blood meal, most females make no attempts to take another. A few females were seen to finish their first meal, run the tarsal spines down the labial groove repeatedly, and then reinsert the fascicle immediately afterwards, but without apparently taking any more blood, even though a streak of blood could be seen in the fascicle.

To determine how soon females would again take blood, they were offered the same forearm at hourly intervals for a 5 min period. As shown in Table II, each took another blood meal 2 to 5 hrs after the first feeding. The above results represent the maximum rate of feeding over a 5 hr period using a highly acceptable donor. The rate of refeeding was strikingly lower with another donor. From the time stage 5- on the engorgement scale is reached until the end of the blood meal and for several hrs thereafter, females excrete fluid, the loss of which allows for further imbibition. One can observe a similar refeeding by interrupting a female's initial blood meal at stage 4 + or 5-. Such females re-land and penetrate and imbibe within less than 1 hr. The variability of the time to locate the arm and the total feeding time for the second meal was strikingly less than for the first (Table II).

Feeding behavior following surgery

To determine some of the many parameters governing the process of blood feeding, a series of surgical operations were performed on females. In the first experiment, females which were previously seen to labellate were removed and anesthetized so that a small portion of the last tarsal segment could be removed from each leg. After recovery, many females tumbled or somersaulted upon landing on an exposed forearm. If they alighted on a flat plane or were tilted uphill, they could feed much more easily and rapidly than if the body was tilted downwards. A few females were seen engaging in a most remarkable act: they hovered close to the arm, and while hovering, actually succeeded in just penetrating the skin. As soon as this happened, they landed and fed normally.

In the second experiment, one or more legs were cut off at the coxae. When only one or 2 legs were removed, feeding on a human forearm was not affected. When 3 legs were amputated, it appeared absolutely necessary for the remaining legs to form a tripod, with the legs being spaced further apart than normal to provide a stable base. The tip of the abdomen was generally lowered to serve as an additional support. After ipsilateral removal of 3 legs, some females placed one of their legs over to the legless side and took a blood meal. A female with 4 legs removed could still take a blood meal, provided that, of the remaining legs, one was in front and one was in the middle on the opposite side of her body. Penetration of the host by such a mosquito did not require a longer time, despite the relatively unstable base of the insect. The tip of the abdomen was used as a support from the time of landing until the mosquito flew away after taking a blood meal.

Various appendages of the head were removed. When the tip of the labella was cut off along with the tip of the fascicles, the mosquitoes would position their proboscides correctly but as their body weights were shifted in an attempt to penetrate the skin, the fascicles slid under their bodies.

When the fascicle was desheathed and only the lobes of the labella were removed, most females did not attempt to probe after landing on the arm. They would land and move the proboscis in small circles, but did not appress the exposed fascicle tips to the skin. A very few attempted to force the fascicle into the skin by pushing the whole body forward and downward. No desheathing or penetration was achieved, however, since the proboscis slid beneath the head.

When the fascicles were desheathed and their tips removed from a group of females, all landed on an exposed forearm but most did not probe. Penetration was not achieved by those which did probe, either due to a forward bending of the fascicle or inability to pierce the skin.

Two methods of artificial desheathing of the fascicle were used. Females desheathed by the MacGregor (1930) technique did not obtain a blood meal. Those attempting to do so, could not penetrate. Their proboscides repeatedly were pressed to the skin, but bent under the females' bodies. Using the loop method of temporary desheathing (Pilitt, 1971), 60% of the females tested were able to obtain a blood meal, but only after resheathing.

The effect of complete darkness on the ability of females to locate and obtain a blood meal was tested at 2200 hrs when the females had been in complete darkness for 2 hrs by inserting a forearm into the cage. All of the females took a blood meal during the 15 min test.

To study whether the antennae are critical to locating a human forearm, both of these sensory appendages were severed as close as possible to Johnston's organ from a group of females. Afterwards, a forearm was offered in a lighted insectary, at which time all of the females took a blood meal. The time required to locate the host was within the range of host-finding times of intact females. Another group of antennectomized females were offered a forearm in a completely darkened insectary at 2200 hrs and none of them took a blood meal within 15 mins.

Response of restrained females to a human forearm

Fasting females were etherized and glued to fine insect pins and after a 3 hr recovery were suspended over a forearm for 5 min, the arm being positioned to approximate an unrestrained feeding area. When the mosquitoes were held lightly against the forearm, some of them labellated and repeatedly attempted to penetrate, but the fascicles slipped off the surface of the skin to one side or the other, and none succeeded in penetrating the skin. When the females were placed on the arm at incorrect or awkward angles, they made no attempts to labellate. All of these non-labellating females had strongly upcurved proboscides after being placed in contact with the arm. It was obvious that even during the most careful artificial positioning, the angles of penetration did not begin to approximate the normal feeding stance of the unrestrained mosquito.

Responses of females to Galleria mellonella *larvae and* Hyalophora cecropia *adults*

Large last stage larvae of *Galleria mellonella,* which were reared at 35° C according to the method of Beck (1960), were placed in a 2-inch container into which fasting females were aspirated. During a 30-minute observation period, all of the mosquitoes approached and frequently landed and rode on the backs of the wriggling larvae and made directional thrusts, labellated, and repeatedly attempted to pierce the cuticle with their desheathed fascicle tips, usually at the intersegment. The mosquitoes were often prevented from penetrating the larvae due to the nearly constant writhings of the latter. One female which succeeded in piercing made only a very shallow insertion before being dislodged.

Larvae used in the preceding test were removed an immersed in a water bath held at 42° C for 5 mins, after which they were placed, without direct handling, on the floor of the cage containing the same group of females. Since the "coddled" larvae were not approached during a 5 min period, they were removed with forceps and lightly skewered with pins and attached to the sides of the screen near the top of the cage. All of the mosquitoes quickly landed on them, labellated, inserted their fascicles easily into the re-located larvae and most of them withdrew varying amounts of hemolymph from the hemocoele. While some females were seen to imbibe hemolymph which had oozed from the wounds made during skewering, most walked away and penetrated the cuticle some distance from the exposed hemolymph. Those females which had taken the most hemolymph were the ones which had re-visited and re-penetrated the larvae.

Restrained adults of both sexes refused to imbibe any hemolymph from freshly heat-fixed larvae when their proboscides were inserted into capillary tubing containing this fluid.

When 2 large adults of *Hyalophora cecropia* were placed in a cage with mosquitoes of both sexes, a few of each landed on the moths' wings and abdomens and both sexes made directional proboscidal thrusts, but the females were never seen to penetrate and obtain hemolymph.

Response of fasted females to recently-blood-engorged mosquitoes

Recently human-blood-fed mosquitoes were introduced into a 1 cu ft cage containing numerous unfed females, and none of the fasting individuals approached the engorged ones during a 1 hr period at 27° C. When females which had just taken a human blood meal were aspirated into a much smaller cage (2 inch diameter) containing fasted females, none of the latter were obviously attracted to the blood-fed mosquitoes, but would occasionally bump into or walk over them and sometimes would rapidly labellate the walls of the cage or make frequent proboscidal thrusts through the screen flooring around them. When a fasted female touched a standing blood-fed individual, the latter would move slightly out of her path or would fly away.

When individual females, whose legs had been removed after blood feeding, were closely confined in very small compartments with unfed females, only 1 out of 20 of the unfed mosquitoes approached the immobilized one and penetrated the blood-distended abdomen and fed.

Since it had been reported that *Aedes aegypti* would rapidly feed on mosquitoes which had recently fed on chick blood (Wetthersby, Hyong-Sun, and McCall, 1971), it was decided to determine whether mosquitoes containing blood of a higher temperature than that of human beings would be more attractive to fasted females. When a week-old chick was held by hand in a cage of females, all of the mosquitoes quickly landed on the hand and none on the chick. When the chick was placed alone in the cage, the mosquitoes landed either on the feathered body or alighted near the eyes or on the legs where some obtained blood. As soon as they had engorged, they were introduced into a small container with fasted females. None of the latter approached or attempted to labellate or pierce the distended females.

The possible effects of higher temperature of imbibed blood on the attractiveness of fed females to fasted individuals were studied by immersing a group of human-blood–fed mosquitoes in a waterbath held at 42° C for 5 mins and quickly placing them in a small cage with fasting females.

During the first 7 mins of observation, the fasted mosquitoes indiscriminately labellated and made directional thrusts through the screening, and when they encountered blood–fed individuals, either avoided them or walked over their motionless bodies without labellating. After 7 mins 20 secs, a single fasted female directly approach and quickly began penetrating the pleura of one of the blood-engorged mosquitoes. Her fascicle was thrust deeply into the abdomen for almost its entire length, and blood could be seen passing from one abdomen to the next. This imbibing female repeatedly partially withdrew her fascicle and then re-inserted it in the same puncture site. This female was approached by two other fasted females which touched her body with their proboscides and immediately began to desheath their fascicles in an attempt to penetrate her abdomen. As they vigorously climbed onto or around her body, they violently probed with their glistening fascicular tips, her head, sides of thorax and abdomen, and especially her wings, but neither succeeded in penetrating. They did not labellate or thrust into the body of the heat-treated mosquito. The original imbiber continued to withdraw blood from the prone body of the heated female while kicking at the probing intruders.

The fascicle of one intruder thrust at the head of the imbibing female, slipped off onto the body of the heat-treated donor mosquito, and was inserted alongside that of the first imbiber, and withdrew blood from the site for a short time, before the displacement of the original imbiber. A third female now approached the heated mosquito and made a separate puncture and briefly imbibed blood and withdrew her fascicle only to return and probe the wings and abdomen of the second imbiber without actually penetrating.

Many of the remaining fasting females now approached 3 previously ignored heat-treated mosquitoes 22 mins after being placed together. They repeatedly probed or made violent thrusts at different parts of them with their labella and fascicular tips. Probing activity seemed concentrated around the pleural regions of blood-filled abdomens. Attempts to penetrate them were almost continuous in spite of frequent failure to achieve insertion. Often during this frenzied activity, females were seen to probe others which were themselves in the act of penetrating the heat-fixed bodies. While no female succssfully penetrated an active mosquito, many successfully punctured the pleura of the heated individuals and subsequently imbibed slowly from them with characteristically frequent partial withdrawals of their fascicles followed by re-penetrations. Often a second female which was actively thrusting would climb over and displace another feeding female and penetrate apparently the identical site. Those which used a new feeding site on the pleura often seemed to have some difficulty in penetrating the cuticle.

At the conclusion of the above observations, it was noted that the one heat-treated female which had been able to recover enough to walk about had not been penetrated, so that she remained full of blood. Only one of the previously fasted females which took blood was engorged, and the remaining imbibers took varying but generally small to moderate amounts.

Discussion

The present studies present a unified view of several major parameters involved in the blood-feeding behavior of a single species of mosquito. Although adult males of *A. aegypti* are clearly greatly attracted to man, their visits, though frequent, are of relatively short duration. While the males often lightly touch their labella to the surface of the skin and may momentarily closely appress them thereto, they were never observed to make any attempts to penetrate the skin. Males directionally thrust and labellate in a manner somewhat similar to the females, but, unlike the females, they lack maxillary stylets for cutting into the skin. Although a few dehydrated males will drink defibrinated blood from open drops, when given a choice between sucrose and blood, they choose the sucrose.

While it has been previously noticed that recently-emerged mosquitoes do not immediately take a human blood meal, broadly divergent data as to the time of her first blood meal are reported for *A. aegypti.* Thus, a range of 18 to 40 hrs is given by 4 different groups (Marchoux, Salimbeni and Simond, 1903; Peryassú, 1908; Mitchell, 1907; and Howard, Dyar and Knab, 1912). According to Macfie (1915), females feed for the first time on the second day, but more often on the third day. In the present work, the majority of the females took their first blood meal when they were 23 to 26 hrs old. Since newly emerged females are generally quiescent and not attracted to man until they are about 1 day old, it may be that host-seeking in females is activated by a hormone.

Although Gordon and Lumsden (1939) observed the raising of the maxillary palps in *Aedes* and recognized that this was the first sign of their intention to feed, they did not specifically correlate this with either insertion or withdrawal of the fascicle. In our study, activities of the palps were found to be directly correlated with specific phases of the feeding act. Thus, raising of the palps occurred whenever the labella were appressed to an acceptable substrate. Initiation of palpal rocking was found to indicate the beginning of maxillary stylet activity associated with fascicular penetration. The sudden cessation of palpal movements signals the presence of blood in the fascicle. The resumption of movement marks the beginning of fascicular withdrawal. Utilizing this information has permitted, for the first time, a truly accurate recording of the times involved in the various phases of blood-feeding (penetration, imbibition and withdrawal).

The excretion of droplets during and at the end of blood-feeding has been observed by Bonne-Wepster and Brug (1932) and Christophers (1960). Although Boorman (1960) observed anal discharges 5 to 15 mins after withdrawal of the fascicle, we have seen excretion occuring over a 2 hr period and probably for much longer, but at an ever-slowing rate.

Robinson (1939) thought that *Anopheles* females required a thrust of the whole body in piercing the human skin, and Christophers (1960) theorized that the legs provide a fulcrum for this action. The present work provides the first direct evidence supporting this theory. The necessity of a fulcrum as opposed to a strong thrust was shown by the severing of various combinations of legs: in all cases, some type of support near the front of the thorax was necessary, whether provided by a remaining prothoracic leg or a mesothoracic leg which was placed further forward. While *A. aegypti* do not make obvious or vigorous forward thrusts at the moment of fascicular insertion, their bodies are inclined forward and downward during penetration. When all of the tarsi were removed so that the height of the mosquito was lowered significantly (presumably this lowering would thereby decrease the power of a leg-initiated thrust), they were all able to pierce the skin and take blood rapidly. However, the loss of either the tarsal tips or the entire tarsi of all the legs reduced the number of their feeding sites and generally forced them to use those flat or slightly inclined areas which did not require grasping. It should be noted, however, that in a few cases a female was able to penetrate a vertical surface of a forearm while hovering over the area and that only after initial fascicular penetration had occurred were all the legs placed on the host and wing activity ceased. Thus, it has been shown that any combination of legs or positioning thereof sufficient to maintain the proboscis at a suitable height above the substrate prior to penetration, without excess pressure on its tip, and sufficient to allow subsequently a slight controlled force to be applied to the fascicle during the penetration period could explain this phenomenon. It is possible, but unproven, that the teeth on the maxillary stylets pull the fascicle into the skin as opposed to being pushed by the body, and thus tilting of the body of the mosquito may merely represent the resultant accomodation to the lowering of the head. The weight of the mosquito's body alone, of course, may exert sufficient force upon the fascicle. The removal of the tarsi presumably decreases the forward thrusting ability of the mosquito, but would not significantly affect the force due to a shift in the position of the body. Thus, it would seem that a fulcrum rather than a thrust is the probable mechanism involved in skin penetration.

Harris and Cooke (1969) stated that *A. aegypti* would land and feed on living *Galleria* larvae, and they suggested that mosquitoes might be able to obtain enough protein from the hemolymph of some insects in nature to develop a few eggs.

While some individuals of our strains rapidly landed on active *Galleria* and vigorously attempted to penetrate them, only one out of 5 succeeded in doing so only momentarily and was dislodged before she could withdraw hemolymph. It appeared that the general failure to achieve penetration was due to the vigorous writhings of the larvae. Harris and Cooke (1969) also found that when *Galleria* had been "coddled" and suspended from the sides of a cage, female mosquitoes landed on them and fed. In the present work, it was observed that if the heat-treated larvae were not suspended, the mosquitoes did not land on them. The probable reason for this may be that specimens of *Galleria* are not very attractive to begin with and were discovered only because they were placed where the mosquitoes frequently landed, that is to say, a mosquito normally lands and rests on the vertical sides of a cage or on the roof and generally does not land or walk on the floor. Suspended heat-treated *Galleria* attracted many females which readily penetrated and withdrew varying amounts of hemolymph. They imbibed more slowly than with human blood. While Harris, Riordan and Cooke (1969) showed a fully engorged *A. aegypti* female still inserted into a *Celerio euphorbiae* larva, our mosquitoes never fully engorged on *Galleria*. Although Harris and Cooke (1969) stated that their *Galleria*-fed mosquitoes were able to lay eggs, our experiences indicate that hemolymph feeding is not an adequate substitute for vertebrate blood.

Weathersby, Hyong-Sun and McCall (1971) discovered that fasting *A. aegypti* were rapidly attracted to female mosquitoes which had just taken a blood meal from a chick. They also noted that the fasting females easily penetrated and withdrew blood from apparently unresisting engorged mosquitoes. Our strains of *A. aegypti* when fed on human blood were definitely not attractive to fasted mosquitoes either in a 1 cu ft cage or in small plastic containers, even under crowded conditions immediately following a blood meal on either a human or a chick. It was further observed that when the engorged females were touched, apparently by chance, by a fasting female, they moved away. Even after removing the legs of human-blood-fed females, they still did not attract fasting females which were relatively close to them and they were not penetrated, even if encountered, unless both the engorged and fasted mosquitoes were very closely confined. However, it was found that if the human-blood-fed females were heated to 42° C prior to being offered to fasting females in a 2 inch cage, they were attractive, but not immediately after being treated. Since it took at least 7 mins before the first apparently directed visit occurred, it is difficult to believe that the blood within the engorged mosquito would have retained its additional heat this long. A probing frenzy elicited in fasting females by the near presence of such heat-treated bodies of the engorged mosquitoes has never been described. The cause of this frenzy remains to be determined.

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SUMMARY

1. General reactions of adult male and female *Aedes aegypti* to human beings in the laboratory are briefly described.

2. Males fly about for a long time in the presence of a human forearm in a cage, and they frequently land and walk about briefly on the skin. Although they touch their labella to the skin, they were seen to attempt to penetrate it.

3. When males were offered a choice between pools of sucrose and defibrinated sheep blood, they drank sucrose.

4. Most adult females take their first blood meal when they are 23 to 26 hrs old. As soon as a feeding site has been located, they lower their hind legs.

5. The behavior of the maxillary palps indicates the major phases of the normal blood-feeding act. When an acceptable substrate is encountered, the palps are suddenly raised. When penetration of the skin starts, the palps begin to move up and down rapidly. As soon as blood appears in the food canal, the palps abruptly stop moving. They begin to move again as soon as the fascicle begins to be withdrawn, and they continue to move until the fascicle is completely removed from the wound.

6. Females always insert their fascicles more slowly than they withdraw them. Engorement on blood varied from 45 to 225 secs. After taking a full blood meal, females will seek and rapidly take another 2 to 5 hrs after the first.

7. Before becoming fully engorged on blood, a female begins to excrete fluid from her anus and continues to do so for 2 hours after completing the meal.

8. Various surgical operations showed that females could take blood when all of their tarsal tips were removed and when all but two of their legs had been amputated. They could not penetrate the skin after their labella were cut off or when the labial sheath was removed, even though they could position themselves correctly on the skin and attempted to pierce.

9. Although females with both antennae removed can locate and feed upon a human forearm in a lighted insectary, they cannot do so in total darkness.

10. Females which had been glued to pins and oriented carefully on a human arm did not penetrate or take blood, probably because the angle for penetration is highly critical and was not approximated.

11. *Aedes* will land on and probe active *Galleria* larvae and *Hyalophora* adults but do not obtain hemolymph from them. Although females can imbibe hemolymph from heat-fixed *Galleria* larvae, they actively reject heat-fixed hemolymph when their proboscides were forced into it.

12. Fasting *Aedes* females did not approach or attempt to probe normally resting or immobilized adults which had just engorged on either a young chick or a human being, except for one case involving very close confinement. Some fasting females were observed to take varying amounts of blood from immobile adults which had been heated to 42° C, but only after a lapse of 7 to 22 mins.

LITERATURE CITED

Beck, S. D., 1960. Growth and development of the greater wax moth *Galleria mellonella* L. *Wisconsin Acad. Sci.*, **49**: 137–148.

Bonne-Wepster, J., and S. L. Brug, 1932. The subgenus *Stegomyia* in Netherland India. *Geneeskundig Tijdschrift Nederlandsch-Indië, Bijblad*, **2**: 35–119.

Boorman, J. P. T., 1960. Observations on the feeding habits of the mosquito *Aedes (Stegomyia) aegypti* (Linnaeus): the loss of fluid after a blood-meal and the amount of blood taken during feeding. *Ann. Trop. Med. Parasitol.*, **54**: 8–14.

CHRISTOPHERS, S. R., 1960. *Aedes aegypti (L.). The Yellow Fever Mosquito.* Cambridge University Press, Cambridge, 739 pp.

GORDON, R. M., AND W. H. R. LUMSDEN, 1939. A study of the behavior of the mouth-parts of mosquitoes when taking up blood from living tissues; together with some observations of the ingestion of microfilaria. *Ann. Trop. Med. Parasitol.,* **33**: 259–270.

HARRIS, P., AND D. COOKE, 1969. Survival and fecundity of mosquitoes fed on insect haemolymph. *Nature,* **222**: 1264–1265.

HARRIS, P., D. F. RIORDAN AND D. COOKE, 1969. Mosquitoes feeding on insect larvae. *Science,* 164: 184–185.

HOWARD, L. O., H. G. DYAR AND F. KNAB, 1912. *The Mosquitoes of North and Central America and the West Indies,* Vol. 1. Carnegie Institution of Washington, Washington, D. C., 520 pp.

MACFIE, J. W. S., 1915. Observations on the bionomics of *Stegomyia fasciata. Bull. Ent Res.,* **6**: 205–210.

MACGREGOR, M. E., 1930. The artificial feeding of mosquitoes by a new method which demonstrates certain functions of the diverticula. *Trans. Roy. Soc. Trop. Med. Hyg.,* **23**: 329–331.

MARCHOUX, E., A. SALIMBENI AND P. L. SIMOND, 1903. La fièvre jaune. Rapport de la Mission Francaise. *Ann. Past. Inst.,* **17**: 665–731.

MITCHELL, E. G., 1907. *Mosquito Life.* Putnam, New York, 281 pp.

PERYASSÚ, A. G., 1908. *Os Culicideos do Brazil.* Inst. de Manguinhos, Rio de Janeiro, 408 pp.

PILITT, D. R., 1971. The feeding behavior of adults of *Aedes aegypti* (Linnaeus) under laboratory conditions. *M.S. thesis, University of Maryland,* 85 pp.

PILITT, D. R., and J. C. JONES, 1972. A qualitative method for estimating the degree of engorgement of *Aedes aegypti* adults. *J. Med. Ent.,* 9: 334–337.

REAUMUR, R. A. F. DE, 1738. *Mémoire pour servir à l'Histoire des Insectes,* Vol. 4. L'Imprimerie, Paris, 636 pp.

ROBINSON, G. G., 1939. The mouth-parts and their function in the female mosquito *Anopheles maculipennis. Parasitology,* **31**: 212–242.

WEATHERSBY, A. B., A. HYONG-SUN AND J. W. MCCALL, 1971. Mosquitoes feeding on engorged mosquitoes. *Mosq. News,* **31**: 110–111.

Reference: *Biol. Bull.*, **145**: 140–149. (August, 1973)

CHEMICAL ACTIVATION OF FEEDING IN THE CARIBBEAN REEF-BUILDING CORAL *MONTASTREA CAVERNOSA*

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Specific feeding activators are being identified for an increasing number of cnidarians from a wide range of habitats. As early as 1942, Pantin demonstrated that individual organic compounds stimulate actions accompanying feeding behavior in *Anemonia sulcata,* and he showed that the presence of food or single organic compounds in the water lowers the threshold of mechanical stimulation necessary to discharge sea anemone nematocysts. After Loomis (1955) demonstrated that glutathione elicits feeding behavior in *Hydra littoralis,* the realization that many other cnidarians also respond to substances present in the tissue fluids of their prey prompted investigators to identify chemical activators for diverse members of the phylum (Lenhoff, 1968 and Lindstedt, 1971a). Whether or not tactile stimuli without chemo-stimuli can induce exploration for nourishment in some species remains unsettled (Lenhoff, 1965), and recent evidence suggests that some organisms may even require two or more activators to produce a complete feeding response (Lindstedt, 1971b and Reimer, 1972).

In this paper we present evidence for activation of feeding in the massive Caribbean reef-building coral *Montastrea cavernosa* (Linnaeus) by single chemical agents, and for differences in receptivity of mouths and tentacles to the amino acids proline, glutamic acid, aspartic acid, and arginine.

Materials and Methods

Montastrea cavernosa is one of the most abundant reef-building corals on the Atlantic coast of Panamá at depths below 1 m. During ecological studies (Porter, 1972 and in preparation), *M. cavernosa* was found to make up as much as 30 per cent of some 10 m^2 plots and to be perhaps the major *in situ* coral contributor by volume and weight of limestone to the permanent reef structure.

M. cavernosa has two distinct morphs in Panamá: a large-polyped variety (average polyp diameter 0.92 cm; expanded tentacle length usually 2× the polyp diameter), and a smaller-polyped form (average polyp diameter 0.48 cm; expanded tentacle length generally $\frac{3}{4}$ the polyp diameter). In addition, the larger variety has protruding polyps and displays wide color variation among colonies, ranging from gray or brown to green, blue, or red. The smaller form invariably has short-necked polyps and is either gray-brown or green-brown in color. Our work was done exclusively on the small-polyped morph. In the laboratory, the larger-polyped form indiscriminately captures and ingests seemingly inert objects ranging from filter paper soaked in distilled water to sterilized sand grains and glass beads.

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Specimens of *Montastrea cavernosa* were gathered from 6–7 meters depth near the Smithsonian Tropical Research Laboratory at Galeta Island, Panamá Canal Zone. The animals were maintained in flowing seawater tables supplied by water passed first through a bed of sand. For chemical activation studies, specimens were transferred while still immersed to a 12 liter aquarium continually refreshed by running seawater at 1 liter/minute and arranged with their polyps 5–8 cm from the surface. Experiments were conducted in subdued artificial light after sunset, as polyps then displayed fullest extension.

Nineteen amino acids, the sugars glucose, fructose, and sucrose, plus pipecolic acid, and the tripeptide glutathione were surveyed for activity at concentrations of 10^{-1} and 10^{-3} M. Pieces of Whatman #1 filter paper 2 mm^2 were soaked in test solutions or plain seawater and presented either to mouths or to tentacles. The treated papers were positioned with forceps several mm above the polyp, and gently dropped onto the target area. Each polyp was observed for 5 minutes following the presentation to determine time and degree of response. Strong feeding activators elicited envelopment of the paper by tentacular folding, deposition of the test paper within the mouth, and full ingestion.

An extended polyp has a planar surface and its ring of tentacles is oriented radially outward. A full envelopment and closure sequence involves folding all tentacles upward and around the paper, ultimately closing tightly at the mouth, leaving no tentacles exposed. A convenient scoring scheme was adopted, based on degree of polyp closure relative to the fully closed state. Stages were designated 0, 1, 2, 3 or 4, corresponding, respectively, to fully extended, $\frac{1}{4}$, $\frac{1}{2}$, $\frac{3}{4}$, or fully closed. Degree of polyp closure was recorded with the elapsed time. Five minutes proves to be a more than adequate observation interval, as most activators produce visible responses within a matter of seconds. Compounds which induced activity during survey experiments were subsequently investigated at greater length.

RESULTS

Table I demonstrates that glutamic acid is by far the most successful feeding activator, causing full envelopment on every trial. Proline and pipecolic acid, a proline analogue, are also important chemical activators, followed by arginine and aspartic acid. The other amino acids produce responses essentially no different from the seawater-soaked control. The amino acids methionine, glycine, alanine, lysine, threonine, tryptophan, valine, serine, and leucine produce only rare, weak responses which never proceed beyond stage 1 of polyp closure. Isoleucine, histidine, phenylalanine, and tyrosine, as well as glucose, fructose, and sucrose, produce no responses at all. Reduced glutathione, a strong activator in many other cnidarians, does not produce full closure in any instance. Although three quarters of the polyps tested show incipient stages of the process, they usually return to a fully extended state within 5 minutes.

To test for anatomical localization of receptivity, filter papers were applied directly to different parts of the coral polyp. Regions tested were the mouth, the tentacles, and the space of ectoderm between those points. Receptivity proves

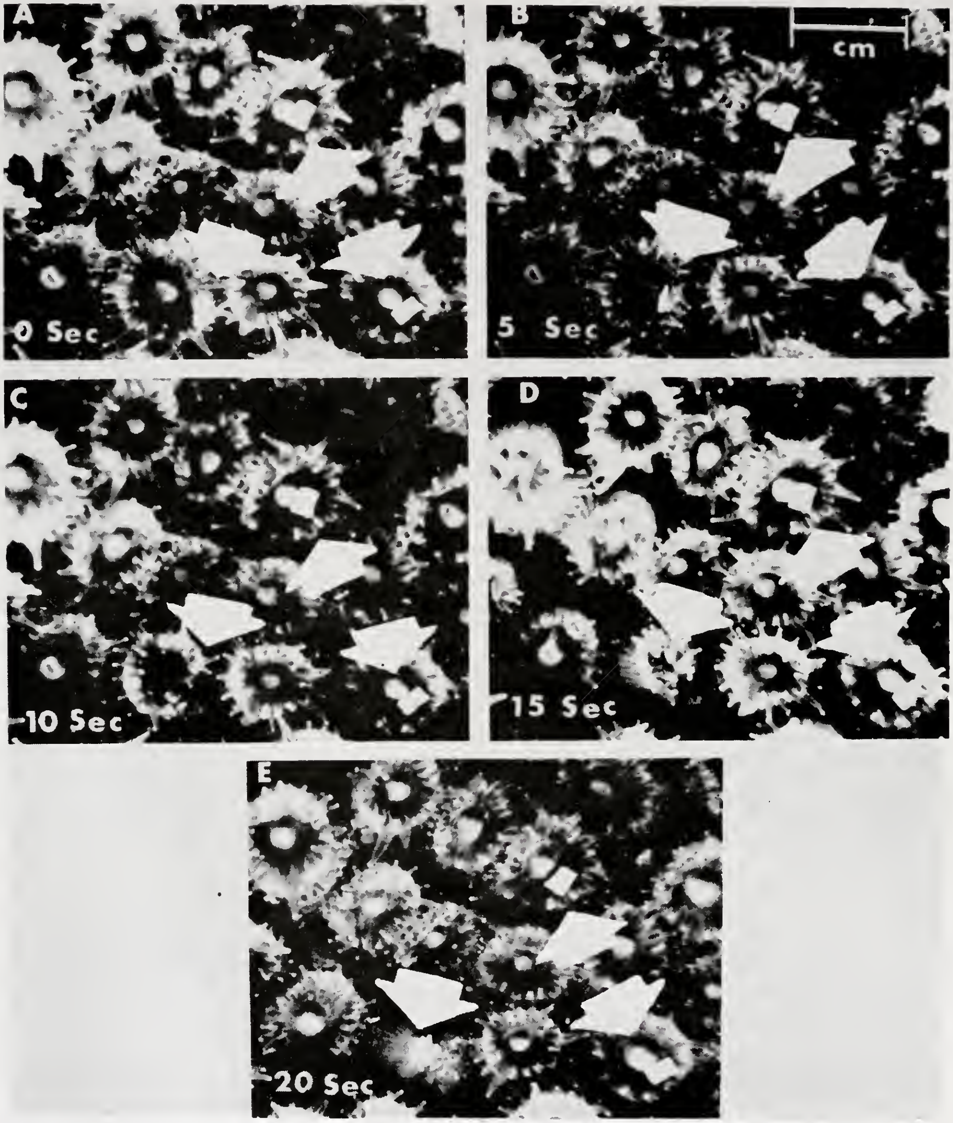

FIGURE 1. Time-lapse photographs of the reactions of *Montastrea cavernosa* polyps to filter paper soaked in 10^{-1} M proline (indicated by left arrow in each photograph) and filter paper controls soaked in seawater (indicated by two right arrows in each photograph). Photograph A shows the coral polyps in State 0, fully open, at the time the three filter papers are dropped. Five seconds later, photograph B shows the polyp with the proline soaked filter paper (left) folding over the paper into State 1, partially closed. Photograph C, ten seconds after dropping, shows the polyp with proline soaked filter paper in State 2; photograph D, 15 seconds after dropping, shows this polyp in State 3; photograph E, 20 seconds after dropping, shows it in State 4, fully closed. Note that the two control polyps (right) do not react to the seawater soaked filter papers. All photographs are 1.5 × life size.

Table I

Compounds tested for activation of feeding in the Caribbean reef-building coral Montastrea cavernosa

Compounds	Molar conc.	Number of trials	Degree of polyp closure							
			1		2		3		4	
			Number of polyps reaching this state	Per cent of trials	Number of polyps reaching this state	Per cent of trials	Number of polyps reaching this state	Per cent of trials	Number of polyps reaching this state	Per cent of trials
Seawater control		107	7	6	2	2				
Glutamic acid	10^{-1}	20	20	100	20	100	20	100	20	100
	10^{-2}	15	9	60	5	33	2	13	2	13
	10^{-3}	12	2	17						
Proline	10^{-1}	29	23	79	19	66	17	59	15	52
	10^{-2}	5	4	80	1	20	1	20		
	10^{-3}	5	1	20						
Pipecolic acid	10^{-1}	29	23	79	18	62	15	52	11	38
	0.05	8	4	50	3	38	3	38	1	12
	10^{-3}	13	3	23	2	15	1	8		
Arginine·HCl	10^{-1}	49	27	55	21	43	19	39	17	35
	10^{-3}	19	2	10						
Aspartic acid	10^{-1}	25	19	76	13	52	8	32	8	32
	10^{-3}	5	0	0						
Glutathione	10^{-1}	16	12	75	2	12				
	10^{-3}	5	0	0						
Cysteine	10^{-1}	11	2	18	1	9				
	10^{-3}	5	0	0						
Asparagine	10^{-1}	16	1	6	1	6				
	10^{-3}	5	0	0						

much greater in the region of the mouth with all activators except glutamic acid and glutathione (Table II). The former compound at 10^{-1} M induces full envelopment and closure whether offered to mouths or tentacles. With 10^{-2} M glutamic acid, however, differentiation between the two sites seems possible, as filter papers produce a stronger response when placed on the tentacles. Neither mouths nor tentacles respond strongly to 10^{-3} M concentrations of glutamic acid. Glutathione yields weak responses regardless of position of application.

Proline, pipecolic acid, glutamic acid, and aspartic acid are not significantly different ($P > 0.1$) in terms of response time. Each produces full closure in 30 seconds or less. All four, however, are distinguishable from arginine, which yields significantly delayed responses ($P < 0.05$). Polyps often remain inactive toward arginine-impregnated filter papers for 1 minute or more before showing the first signs of tentacle movement. Once begun, the envelopment process is usually deliberate and complete.

When a filter paper treated with chemical activator is applied to a polyp already containing an untreated paper, the polyp almost invariably closes only around the treated paper, leaving the control paper exposed. If a distasteful compound is offered, such as 10^{-1} M HCl, the polyp folds immediately, leaving the filter paper outside.

Discussion

Mariscal and Lenhoff (1968) report proline and reduced glutathione to be principal activators in *Cyphastrea ocellina*, an Indo-Pacific coral species, and they find that solutions of these compounds effect mouth opening and tentacle contraction in several other Hawaiian corals. Goreau (1961) indicated that methionine added to seawater causes some Caribbean corals to extrude mesenterial filaments, but that glutathione has little effect on the same animals. Goreau *et al.* (1971) discovered that low concentrations of glycine, alanine, phenylalanine, and leucine trigger a typical feeding response, including extension of tentacles, swelling of the coenosarc, and sometimes extrusion of mesenterial filaments, in the Caribbean corals *Manicina areolata, Eusmilia fastigiata, Isophyllia sinuosa, Mussa angulosa,* and *Scolymia lacera.* Of all these compounds previously implicated in activation studies on corals, only proline elicited predictable feeding behavior in our work on *Montastrea cavernosa.*

Aspartic acid and glutamic acid both elicit feeding responses in *M. cavernosa*, but the structurally analogous asparagine does not. Glutathione a tripeptide consisting of glutamic acid, cysteine, and glycine, likewise fails to produce strong responses. This suggests that the β and γ carboxyl moieties of these compounds are instrumental in the chemical activation sequence and that modification of their terminal acid functions by amide or peptide formation would be expected to diminish activity. Such lower responses are indeed observed with asparagine and glutathione. Strong feeding responses to glutamic acid have been noted in other organisms, including the anemone *Actinia equina* (Steiner, 1957). The greater response to glutamic acid than to aspartic acid in *M. cavernosa* may possibly be ascribed to structural differences between the two, *i.e.* an additional methylene group in the functional side chain of the former molecule. However, aspartic acid (10^{-1} M) shows strongest activity in the mouth region, whereas a single series of tests performed with 10^{-2} M glutamic acid points to the tentacles as sites of greatest glutamic acid receptivity. Such results would not be excepted if the two analogues stimulate the same types of chemorecepors, and imply instead the the existence of separate receptor sites capable of distinguishing between the two molecules. Differential sensitivity between mouth and tentacles may be a general observation in sea anemones (Ross, 1966). Pantin and Pantin (1943) report that the mouth of *Anemonia sulcata* is more sensitive to a wider variety of chemical stimuli than are the tentacles, a pattern repeated in *M. cavernosa* with regard to amino acid sensitivity, as in *Calliactis polypus* (Reimer, 1973).

The response to proline and pipecolic acid suggests that *M. cavernosa* possesses a second type of receptor sensitive to these α-imino acids similar to those demonstrated in *Cordylophora* by Fulton (1963).

An explanation of the response to arginine is largely conjectural. Perhaps a solution of 10^{-1} M arginine may be so concentrated as to inhibit feeding at first, but dilutions to 10^{-3} M provoke no responses worthy of note. Mariscal and Lenhoff (1968) describe a response delay to the proline analogue pipecolic acid in *Cyphastrea ocellina* similar to the lag experienced by us with arginine in *M. cavernosa.* Before responding to pipecolic acid, *Cyphastrea* commonly requires a 1–2 minute lag period, an interval identical to that we found in *M. cavernosa.* Mariscal and Lenhoff suggest the possibility of enteroreception somewhat similar to that discovered in *Hydra* for tyrosine (Blanquet and Lenhoff, 1968). The lag, then, would be the time required for the activator to diffuse into the gastrovacular cavity before the polyp could respond.

Several recent studies performed on sea anemones show that different phases of feeding behavior may be controlled by individual chemical inducers. Lindstedt (1971b) demonstrates that asparagine induces tentacular bending in *Anthopleura elegantissima,* and that glutathione controls the act of swallowing by the anemone once contact with its oral region is made. She finds that aspartic acid also elicits some activity in the tentacle response, but glutamine and glutamic acid are comparatively inactive. On the other hand, it is the γ-glutamyl moiety of glutathione that appears to be most vital to the oral response. Nagai and Nagai (1973), working with the congeneric *A. midorii,* discovered that amino acids mixed with α-starch and water could serve as an artificial feeding inducer for the organism. Alanine, glycine, and histidine each evoke tentaculation; proline produces mouth opening; and cysteine or glutathione effects ingestion.

Williams (1972) similarly separates feeding and pre-feeding responses in *Diadumene luciae.* The pre-feeding, or tentacular, response is stimulated by the presence of certain chemicals in solution, including proline, glutamic acid, and aspartic acid. Filter papers imbibed with glutamic acid, for instance, stimulate only tentacular action, but not ingestion, in *D. luciae.* The oral feeding response in this anemone is most strongly activated by glutathione, several amino acids, and some vitamins of the B-complex.

Separation of oral and tentacular activators may not be as marked in *M. cavernosa* as in the anemones studied, but Tables I and II provide evidence for some such mechanism operating in this coral. Concentrations of 10^{-2} M glutamic acid strongly evoke the early stages of tentacle bending, as does aspartic acid when it is specifically applied to the tentacles. Proline and arginine activity, however, is rather more confined to the mouth region. Such observations are consistent with the reported general behavior of biphasic feeding activators. Glutathione also seems to stimulate the very early stage of tentacular bending, but since full ingestion never follows with this compound, it is difficult to say whether or not the response constitutes an authentic feeding activation. Forrest (1962) shows, for instance, that while glutathione induces prolonged mouth opening and tentacle contraction in *Hydra,* it does not duplicate, and can even inhibit, the natural feeding behavior of the animal. Despite its implication in mouth opening and tentacle contraction as evidence of chemical activation in Hawaiian corals (Mariscal and Lenhoff, 1968), glutathione does not trigger any responses in *M. cavernosa* that could be unhesitantly classed with the envelopment sequences induced by several amino acids.

M. cavernosa is a particularly voracious predator; its feeding response has been observed in the field where naturally occurring concentrations of net zooplankton at times exceed 2.0 mg dry wt m^{-3} (Porter, in press). Capture of live prey by the coral depends largely upon random collisions of zooplankton with extended polyps, inducing nematocyst discharge and subsequent envelopment. Mariscal and Lenhoff (1968) suggest that the puncture of a zooplankter by coral nematocysts causes the release of chemical feeding activators into the water. These authors and others (A. A. Reimer, Biology Department, Pennsylvania State University, University Park, Pennsylvania, personal communication) tested the hypothesis on Pacific species, using polyp mouth opening as a criterion of activator success. The size and voracity of the *M. cavernosa* polyp, however, permit us to duplicate a full capture and ingestion response in the laboratory by using filter papers impregnated with strong feeding activators.

Free amino acids are of equal importance with inorganic ions in the osmoregula-

TABLE II

Compounds tested for localized activation of feeding in the Caribbean reef-building coral Montastrea cavernosa

Compounds	Molar conc.	Number of trials	Degree of polyp closure							
			1		2		3		4	
			Number of polyps reaching this state	Per cent of trials	Number of polyps reaching this state	Per cent of trials	Number of polyps reaching this state	Per cent of trials	Number of polyps reaching this state	Per cent of trials
Proline	10^{-1}									
on mouth		11	11	100	11	100	11	100	10	91
on tentacles		9	4	44	1	11	1	11	1	11
between		9	8	89	7	78	5	56	4	44
Pipecolic acid	10^{-1}									
on mouth		8	8	100	8	100	7	88	7	88
on tentacles		12	8	67	3	25	2	17	2	17
between		9	7	78	7	78	6	67	2	22
Arginine·HCl	10^{-1}									
on mouth		16	11	69	11	69	11	69	10	62
on tentacles		21	7	33	4	19	4	19	4	19
between		12	9	75	6	50	4	33	3	25
Aspartic acid	10^{-1}									
on mouth		12	10	83	8	67	7	58	7	58
on tentacles		11	9	82	4	36				
between		2	2	100						
Glutamic acid	10^{-2}									
on mouth		3	0	0						
on tentacles		9	7	78	5	56	2	22	2	22
between		3	2	67	2	67				
Glutathione	10^{-1}									
on mouth		6	4	67	1	17				
on tentacles		6	4	67	1	17				
between		4	4	100						

tion of crustacean tissue fluids. The six most prominent amino acids reported for members of the class are alanine, arginine, glutamic acid, proline, glycine, and and taurine (Huggins and Munday, 1968). Johannes and Webb (1965) report that living marine zooplankton release amino acids into solution at a rate that increases with water temperature. In warm seas, as much as 30 mg of α-amino nitrogen per gram dry wt of zooplankton per day can apparently diffuse from the animals into the surrounding medium. The composition of the amino acid assemblage varies among individual zooplankton populations.

Cowey and Corner (1963) report that the free amino acid pool of the copepod *Calanus finmarchicus* equals 16 to 20 per cent of the protein amino acid content of the animal. Glycine, taurine, and arginine are major contributors to the pool, but proline and glutamic acid constitute 9.29 and 3.20 μM/g wet wt, respectively. Raymont *et al.* (1968) discovered that free amino acids, chiefly glycine, alanine, and glutamic acid, comprise most of the non-protein nitrogen, amounting to 20 per cent of total cell nitrogen, in *Neomysis integer*, a temperate, shallow-water mysid shrimp. Srinivasagam *et al.* (1971) find that glycine, taurine, and arginine comprise 70 per cent and glutamic acid, proline, and alanine form 15 per cent of the free amino acids of the same organism. These investigators find individual amino acid concentrations in excess of 1 mg/g dry wt in all three zooplankton species they measured. In *Sphaeroma rugicauda,* Harris (1969) measured 2.10 mg proline/100 mg dry wt of the isopod, and quantities of arginine, aspartic acid, and glutamic acid roughly one third this value. Simpson, Allen and Awapara (1959) find 2 μM free glutamic acid and 6 μM arginine per gram of live decapod tissue, and Emerson (1967) reports the free amino acids proline, glutamic acid, and aspartic acid present in *Artemia salina* embryos at more than 1 μg/mg dry weight. All of these figures correspond to 10^{-4} to 10^{-2} M concentrations of the individual amino acids in crustacean tissue fluids, concentrations in the range we observe for excitation of feeding behavior. Zooplankton punctured by nematocysts might be expected to leak their contents into the water near receptor sites, but any direct comparison of these values with concentrations tested by us must take into account dilution from the filter paper, diffusion rates, and ultimate concentration of activator in the microenvironment near chemoreceptors.

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SUMMARY

1. Nineteen amino acids, pipecolic acid, glutathione, glucose, fructose, and sucrose were surveyed as potential chemical activators of feeding in the massive Caribbean reef-building coral *Montastrea cavernosa.*

2. Glutamic acid, proline, pipecolic acid, arginine, and aspartic acid active feeding in this species. Its polyps fully ingest pieces of filter paper impregnated with these compounds.

3. Glutamic acid, proline, pipecolic acid, and aspartic acid produce full envelopment in 30 seconds or less. Arginine, however, requires one minute or more before eliciting closure.

4. Sensitivity is greater at the mouth for proline, aspartic acid, and arginine, but greater on the tentacles for glutamic acid.

5. *Montastrea cavernosa* responds to compounds of several chemical structural groups and therefore possibly has different chemoreceptors sensitive to each of these groups.

6. Since a variety of crustacean zooplankton have been shown to contain comparable concentrations of some of these activators, the release of such compounds following puncture of zooplankton by coral nematocysts may elicit the observed capture and ingestion behavior in *Montastrea cavernosa.*

LITERATURE CITED

Blanquet, R. S., and H. M. Lenhoff, 1968. Tyrosine enteroreceptor of *Hydra*: its function in eliciting a behavior modification. *Science,* **159**: 633–634.

Cowey, C. B., and E. D. S. Corner, 1963. Amino acids and some other nitrogenous compounds in *Calanus finmarchicus. J. Mar. Biol. Ass. U.K.,* **43**: 485–493.

Emerson, D. N., 1967. Some aspects of the free amino acid metabolism in developing encysted embryoes of *Artemia salina* the brine shrimp. *Comp. Biochem. Physiol.,* **20**: 245–261.

Forrest, H., 1962. Lack of dependence of the feeding reaction in *Hydra* on reduced glutathione. *Biol. Bull.,* **122**: 343–361.

Fulton, C., 1963. Proline control of the feeding reaction of *Cordylophora. J. Gen. Physiol.,* **46**: 823–838.

Goreau, T. F. 1961. Comment in H. M. Lenhoff, Activation of the feeding reflex in *Hydra littoralis.* Pages 230–231 in H. M. Lenhoff and W. F. Loomis, Eds., *The Biology of Hydra.* University of Miami Press, Miami.

Goreau, T. F., N. I. Goreau and C. M. Yonge, 1971. Reef corals: Autotrophs or heterotrophs? *Biol. Bull.,* **141**: 247–260.

Harris, R. R., 1969. Free amino acid and haemolymph concentration in *Sphaeroma rugicauda* (Isopoda) during adaptation to a dilute salinity. *J. Mar. Biol. Ass. U.K.,* **50**: 319–326.

Huggins, A. K., and K. A. Munday, 1968. Crustacean Metabolism. Pages 271–378 in O. Lowenstein, Ed., *Advances in Comparative Physiology and Biochemistry,* Vol. 3. Academic Press, Inc., New York.

Johannes, R. E., and K. L. Webb, 1965. Release of dissolved amino acids by marine zooplankton. *Science,* **150**: 76–77.

Lenhoff, H. M., 1965. Mechanical stimulation of feeding in *Epiactis prolifera. Nature,* **207**: 1003.

Lenhoff, H. M., 1968. Chemical perspectives on the feeding response, digestion, and nutrition of selected coelenterates. Pages 157–221 in M. Florkin and B. T. Scheer, Eds., *Chemical Zoology,* Vol. 2. Academic Press Inc., New York.

Lindstedt, K. J., 1971a. Chemical control of feeding behavior. *Comp. Biochem. Physiol.,* **39A**: 553–581.

LINDSTEDT, K. J., 1971b. Biphasic feeding response in a sea anemone: control by asparagine and glutathione. *Science*, **173**: 333-334.

LOOMIS, W. F., 1955. Glutathione control of the specific feeding reactions of *Hydra*. *Ann. New York Acad. Sci.*, **62**: 209–228.

MARISCAL, R. N., AND H. M. LENHOFF, 1968. The chemical control of feeding behavior in *Cyphastrea ocellina* and in some other Hawaiian corals. *J. Exp. Biol.*, **49**: 689–699.

NAGAI, Y. AND S. NAGAI, 1973. Feeding factors for the sea anemone *Anthopleura midorii*. *Mar. Biol.*, **18**: 55–60.

PANTIN, C. F. A., 1942. The excitation of nematocysts. *J. Exp. Biol.*, **19**: 294–310.

PANTIN, C. F. A., AND A. M. P. PANTIN, 1943. The stimulus to feeding in *Anemonia sulcata*. *J. Exp. Biol.*, **20**: 6–13.

PORTER, J. W., 1972. Patterns of species diversity in Caribbean reef corals. *Ecology*, **53**: 745–748.

PORTER, J. W., in press. Zooplankton feeding by the Caribbean reef-building coral *Montastrea cavernosa*. *Second International Symposium on Coral Reefs, Australia*, in press.

RAYMONT, J. E. G., J. AUSTIN AND E. LINFORD, 1968. Biochemical studies on marine zooplankton. V. The composition of the major biochemical fractions in *Neomysis integer*. *J. Mar. Biol. Ass. U.K.*, **48**: 735–760.

REIMER, A. A., 1972. Glutathione-proline activation of feeding behavior in the zoanthid *Palythoa psammophilia* Walsh & Bowers. *J. Exp. Mar. Biol. Ecol.*, **10**: 177–181.

REIMER, A. A., 1973. Feeding behavior in the sea anemone *Calliactis polypus* (Forskål, 1775). *Comp. Biochem. Physiol.*, **44A**: 1289–1301.

ROSS, D. M., 1966. The receptors of the Cnidaria and their excitation. *Symp. Zool. Soc. London*, **16**: 413–418.

SIMPSON, J. W., K. ALLEN AND J. AWAPARA, 1959. Free amino acids in some aquatic invertebrates. *Biol. Bull.*, **117**: 371–381.

SRINIVASAGAM, R. T., J. E. G. RAYMONT, C. F. MOODIE AND J. K. B. RAYMONT, 1971. Biochemical studies on marine zooplankton. X. The amino acid composition of *Euphausia superba*, *Meganyctiphanes norvegica*, and *Neomysis integer*. *J. Mar. Biol. Ass. U.K.*, **51**: 917–925.

STEINER, G., 1957. Über die chemische Nahrungswahl von *Actinia equina* (L.). *Naturwissenschaften*, **44**: 70–71.

WILLIAMS, R. B., 1972. Chemical control of feeding behavior in the sea anemone *Diadumene luciae* (Verrill). *Comp. Biochem. Physiol.*, **41A**: 361–371.

Reference: *Biol. Bull.*, **145**: 150–158. (August, 1973)

FEEDING BEHAVIOR IN POLYPS OF THE CHESAPEAKE BAY SEA NETTLE, *CHRYSAORA QUINQUECIRRHA* (DESOR, 1848)

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The medusae of *Chrysaora quinquecirrha* are familiar to fisherman and swimmers in the Chesapeake Bay area, both for their painful stings and for the huge populations extant during the summer months (Cargo and Schultz, 1966). Most of the life cycle of *Chrysaora,* however, is spent as a sessile polyp, 0.5 to 3 mm in length. In their natural environment, the polyps feed on a variety of small invertebrates and fish (Cargo and Schultz, 1966) which they capture with the nematocysts, or stinging organelles, present in their tentacles. Their feeding behavior is similar to that observed in *Hydra* (Lenhoff, 1961) and in a number of other coelenterates (Fulton, 1963; Mariscal and Lenhoff, 1968; Lindstedt, 1971; Reimer, 1971; Williams, 1972) and can generally be described as follows. Upon stimulation the tentacles contract and bend towards the mouth. The mouth then opens and, in most cases, the tentacles pass through the mouth opening and enter the gut cavity. The latter situation is described in this paper as tentacle stuffing and is shown in Figure 1.

It has long been known that food extracts can elicit such behavior in coelenterates (Nagel, 1892). In 1955, Loomis demonstrated that the tripeptide, reduced glutathione, specifically stimulated the feeding response in *Hydra.* Since that time the amino acids proline (Lenhoff, 1968; Reimer, 1971), valine (Lindstedt, Muscatine and Lenhoff, 1968), asparagine (Lindstedt, 1971), glutamine (Lenhoff, 1968; Williams, 1972), alanine (Reimer, 1971), serine (Williams, 1972), aspartic acid (Williams, 1972), histidine (Williams, 1972), tryptophan (Williams, 1972), and lysine (Reimer, 1971), acting either alone or in combination with reduced glutathione (Lenhoff, 1968; Lindstedt, 1971; Reimer, 1971), have been cited as specific activators of the feeding response in several other coelenterates. In addition, tyrosine, when present in the gut of *Hydra,* causes a modification of the normal feeding response (Blanquet and Lenhoff, 1968).

Information about the chemical nature of specific feeding response activators in coelenterates has generally concerned the classes Hydrozoa and Anthozoa (Lenhoff, 1968) although Muscatine (Lenhoff, H. M., University of California at Irvine, personal communication) has found that reduced glutathione induces feeding behavior in polyps of the scyphozoan, *Aurelia.* The data presented here for the polyp stage of *Chrysaora quinquecirrha* is the first comprehensive study of artificially induced feeding behavior in the class scyphozoa. It is reported in this paper that most naturally occurring amino acids and several small peptides elicit feeding movements in *Chrysaora* polyps. Studies were undertaken to determine

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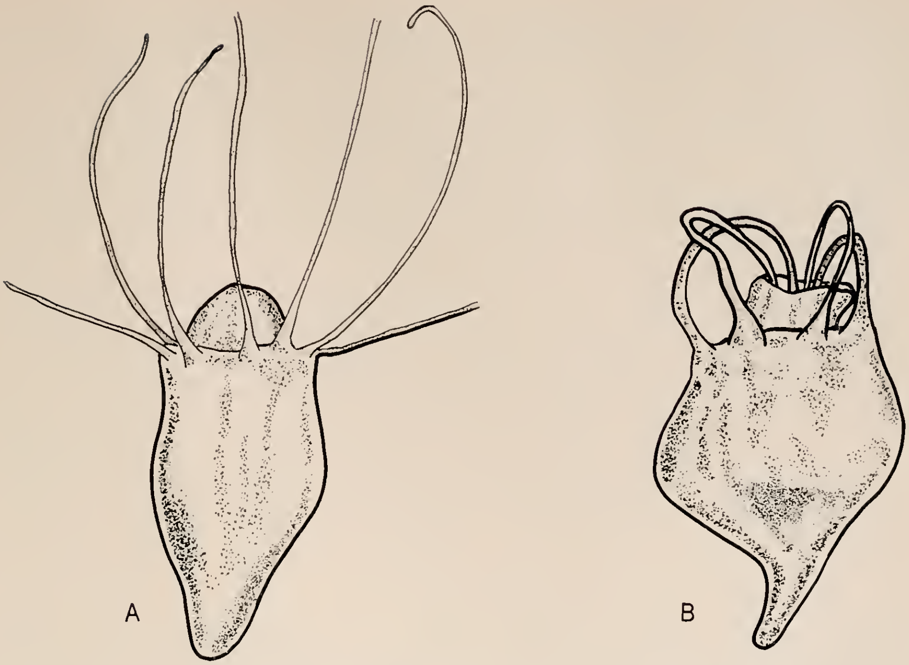

FIGURE. 1. Feeding behavior in *Chrysaora* polyps; (A.) polyp with closed mouth and outstretched tentacles prior to introduction of feeding stimulant; (B.) polyp after exposure to 10^{-5} Molar reduced glutathione. Most of the tentacles have been omitted from the drawings for clarity.

the characteristics of *Chrysaora's* feeding receptor system and the active sites on the amino acids and peptide molecules which stimulate the receptors to activate the pattern of feeding behavior.

MATERIALS AND METHODS

Laboratory cultures of polyps of *Chrysaora quinquecirrha* known as Type 2 (Loeb, 1972) were used in these studies. Artificial seawater (Instant Ocean, Aquarium Systems Inc. Wickliffe, Ohio), prepared as a solution of 13 parts per thousand salt, served as the culture medium. All substances tested were dissolved in and subsequently diluted with this medium; they were initially prepared as 10^{-4} M solutions to approximate the concentration of amino acids in crustacean hemolymph (Florey, 1966; Srinivasagam, Raymont, Moodie and Raymont, 1971). Amino acids, sugars, and reduced glutathione were obtained from Sigma Biochemicals, St. Louis, Mo. Substituted amino acid analogues were from Nutritional Biochemicals, Cleveland, Ohio; ethanolamine and ethylene diamine were from Matheson, Coleman and Bell Inc., Norwood, Ohio; urea from Schwartz-Mann Research Laboratories, Orangeburg, New York, acetaldehyde and valeraldehyde from Eastman Kodak, Rochester, New York. Trypsin and bacitracin were from Calbiochem, Los Angeles, California.

Brine shrimp extract was prepared by homogenizing 100 recently hatched (24 to 48 hours old) shrimp nauplii in a few drops of artifical seawater with the aid of a hand held glass microhomogenizer. Following dilution to 1 ml with artificial seawater, the homogenate was centrifuged 15 minutes at 5000 rpm; the clear supernatant was serially diluted with artificial seawater for use in subsequent experiments. In order to correlate the amino acid concentration of brine shrimp homogenate to the amino acid and glutathione solutions tested, several assumptions were made concerning the homogenate. Several sources (Florey, 1966; Srinivasagam *et al*, 1971) report the amino acid content of the body fluids of small crustacea to be about 10^{-4} M. The weight of the average 24 hour brine shrimp nauplius was determined to be 7.2 ± 0.8 s.d. micrograms. This was accomplished by weighing six individual lots of ten well drained brine shrimp nauplii on a Cahn electrobalance, and then calculating the mean weight of one nauplius. Assuming that the density of each brine shrimp was one and that each brine shrimp was constructed solely as a container holding 10^{-4} M amino acid solution, the concentration of any dilution of brine shrimp homogenate could be roughly estimated. The protein content of the supernatant was determined by the method of Lowry, Rosebrough, Farr and Randall (1951), using bovine serum albumin as the stanard protein.

For each test five *Chrysaoroa* polyps, starved for one week prior to use, were removed from a stock culture and placed in approximately 5 ml of test solution in a small stender dish. Observations were begun immediately with the aid of a dissecting microscope. Each test was repeated one or more times. All work was done at room temperature (23–25° C). Since most of the time taken by feeding behavior involved insertion of tentacles into the mouth and thence into the body cavity (stuffing), feeding time was defined as the time from initial tentacle insertion to complete tentacle withdrawal. Other effects such as tentacle writhing or unusually wide mouth opening (gaping) were recorded as observations. Complete tentacle withdrawal was, in most cases, accompanied by mouth closing and was therefore considered the end of the response. On several testing occasions particularly sensitive polyps exhibited slight feeding responses when dropped into fresh artificial seawater; this time was therefore subtracted from feeding times observed under experimental conditions, and does not appear in the data.

In order to obtain an insight to the location of receptors involved in the feeding behavior patterns, tentacles were cut from two or more animals within 30 seconds after their immersion in the test solution and observed in the same dish as the whole polyps. Tentacles removed in seawater and then placed in minimally effective concentrations of glutathione,. tyrosine, phenylalanine, glutamine and cystein gave the same responses as tentacles cut and observed in these solutions.

RESULTS

General feeding responses were noted for all amino acids tested except lysine. It should be noted that the intensity and duration of the whole response varied with the substance tested; in addition, the intensity of individual components of the response varied. Thus, in some instances, the tentacles would writhe vigorously prior to bending towards the mouth. The mouth might open slightly or gape widely. The extent and duration of tentacle stuffing was dependent on the stimulating substance. The minimal effective concentration for amino acids or small peptides ranged from 10^{-4} M to 10^{-12} M, depending on the chemical being tested. These results are presented in Table I. A commercial protein hydrolysate (Bacto-Peptone, Difco Laboratories, Detroit, Mich.), also induced feeding behavior at a minimum concentration of 0.02 mg/ml (approximately 2×10^{-4} M amino acids if all of the hydrolysate is assumed to be amino acids). No feeding response was observed in the presence of large peptides such as bacitracin (m.w. 1400) (Sober, 1968) or to proteins such as bovine serum albumin or trypsin.

Isolated tentacle responses reflected whole animal responses; tentacle writhing or contraction or elongation occurred in most amino acid and peptide solutions, as noted in Table I. In contrast, control tentacles excisted in fresh medium showed little or no contraction and, propelled by ciliated cells (Chuin, 1930), slowly moved about the dish. Tentacles exposed to a noxious substance such as weak hydrocloric acid merely contracted.

Chrysaora polyps also exhibit the feeding response when presented with a cell-free extract of brine shimp nauplii. However, the characteristics of the response are dependent on the concentration of the extract in the test solution. At

TABLE I

Effect of amino acids and peptides on feeding behavior. The symbols may be interpreted as follows: W, tentacle writhing; S, tentacle stuffing; E, tentacle elongation; C, tentacle contraction; G, gaping mouth; O, no effect; (—), test not performed

	Effect (whole animal)	Effect (excised tentacle)	Minimum effective concentration	Effective time at lowest concentration (minutes)
Alanine	W, G, S	C	10^{-8}	30
Arginine	S	O	10^{-4}	15
Asparagine	G, S	—	10^{-4}	15
Aspartic acid	G, S	—	10^{-4}	10
Cystein	G, S	O	10^{-8}	25
Glutamine	W, G, S	W	10^{-8}	30
Glutamic acid	W, G, S	E	10^{-8}	60
Glycine	S	—	10^{-6}	15
Histidine	G, S	E	10^{-8}	35
Isoleucine	G, S	—	10^{-4}	60
Leucine	S	—	10^{-4}	15
Lysine	O	—	—	—
Methionine	S	W	10^{-6}	35
Phenylalanine	G, S	W	10^{-4}	40
Proline	W, G, S	W	10^{-8}	20
Serine	S	—	10^{-4}	15
Threonine	S	W	10^{-8}	25
Tryptophan	G, S	E, W	10^{-8}	55
Tyrosine	G, S	E	10^{-4}	15
Valine	W. S	W	10^{-8}	50
Glutathione (GSH)	W, G, S	See text	10^{-12}	15
Glycylglycine	G, S	E	10^{-8}	35

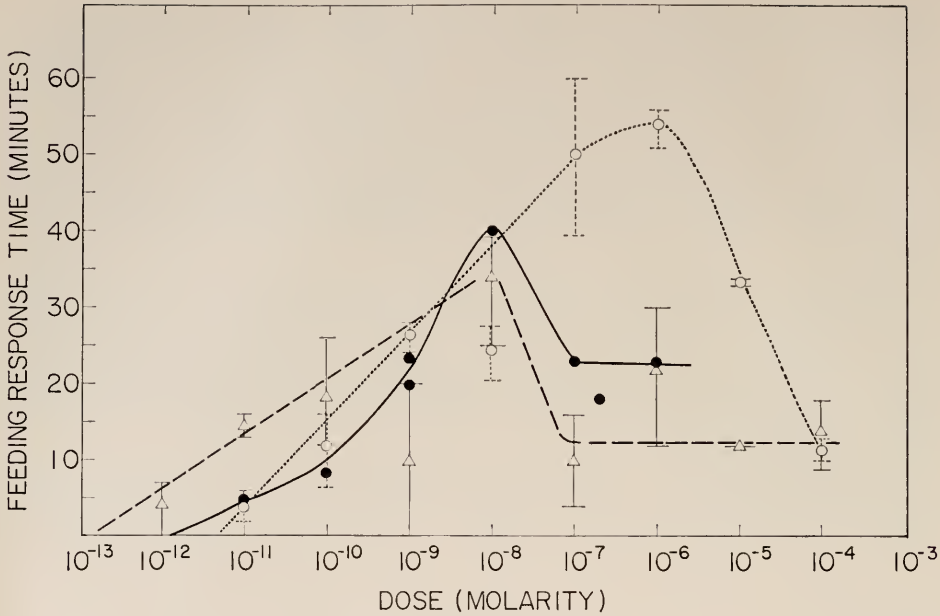

FIGURE 2. Differences in feeding response time as the concentration of feeding behavior stimulus is changed. Open symbols equal response to reduced glutathione. Bars represent the spread in data; each symbol represents the mean feeding time for 10 polyps. Closed symbols equal response to brine shrimp homogenate supernatant. Each symbol represents the mean feeding time for 10 polyps. Data spread omitted for clarity. The dose represents the approximate amino acid concentration of shrimp homogenate and the specific concentrations of the glutathione solutions.

high concentrations (about 10^{-6} M amino acids or 13γ of protein per ml of test solution) tentacle writhing, mouth opening, and tentacle stuffing occurred simultaneously. At somewhat lower concentrations (approximately 10^{-7} to 10^{-9} M amino acids) the onset of tentacle writhing was delayed for a few seconds after the polyps were introduced to the test solution. Mouth opening and tentacle stuffing took place from one to three minutes later. At even lower concentrations of extract (approximately 10^{-9} to 10^{-12} M amino acids), writhing began one to three minutes after exposure to the test solution, mouth opening one to 10 minutes after exposure, and tentacle stuffing 12 to 24 minutes after exposure. In contrast, reduced glutathione elicited simultaneous tentacle writhing, mouth opening and tentacle insertion at all active concentrations.

As shown in Figure 2, the feeding response time increases as the concentration of extract increases, from a concentration of approximately 10^{-12} M amino acids to approximately 10^{-8} M amino acids, and then falls off to lower response times as the concentration of extract increases further. Similar curves were generated by testing known concentrations of reduced glutathione, and are also shown in Figure 2. All polyps were starved for one week prior to testing; each point on the curve represents the mean feeding response time of 10 polyps.

It is interesting to note that isolated tentacles placed into 10^{-4} and 10^{-5} M reduced glutathione are inactive. However, writhing does occur in glutathione solutions of 10^{-6} to 10^{-10} M, indicating that the response is inhibited at higher concentrations of reduced glutathione.

All common amino acids, with the exception of lysine, induce feeding behavior. Proline, glutamic acid and alanine prepared with acetylated α amino groups, and proline, alanine and tyrosine prepared with esterified α carboxyl groups, as well as the substituted amino acid thioproline, all elicited feeding behavior with feeding times comparable to their natural counterparts. The sugars glucose and glucosamine at 10^{-4} M produced no feeding behavior. The amino alcohol, ethanolamine, or the diamine, ethylenediamine, at 10^{-2} to 10^{-4} M, produced no response. Aldehydes such as acetone, acetaldehyde, and valeraldehyde elicited no feeding responses. However, 10^{-3} M urea produced a mean stuffing response time of 42 minutes; 10^{-4} M urea elicited stuffing for a mean time of 30 minutes. However, acetone produced no effect. Some organic acids also induced feeding; α ketoglutarate, 10^{-4} M at pH 7 elicited feeding for 15 minutes; pyruvate and lactate at 10^{-5} M elicited feeding for 30 minutes. Succinate induced no feeding behavior at 10^{-4} M.

DISCUSSION

Chrysaora polyps initiate feeding behavior in response to most naturally occurring amino acids and to some small peptides. This is surprising, since most other coelenterates previously studied respond to only one or two specific compounds (Lenhoff, 1961, 1968; Mariscal and Lenhoff, 1968; Reimer, 1971; Lindstedt, 1971). However, Forrest (1962) suggests multiple feeding activators in several species of *Hydra*, and Goreau, Goreau and Yonge (1971) present evidence that at least four amino acids (glycine, alanine, phenylalanine and leucine) in concentrations as low as 10^{-9} M induce feeding behavior in several species of reef coral. Williams (1972) also showed that some expression of feeding behavior occurs in the sea anemone *Diadumene* in response to six amino acids, reduced glutathione, pyridoxine, and nicotinic acid. However, the concentrations of these chemicals needed to evoke feeding behavior were, with the exception of aspartic and glutamic acids, in the order of 10^{-3} to 10^{-1} Molar. *Chrysaora* responded to activating substances at concentrations of 10^{-4} M or less, responding to reduced glutathione at 10^{-12} M. The concentration of free amino acids in the body fluids of small crustacea, common prey of *Chrysaora* polyps, is about 10^{-4} M (Florey, 1968; Srinivasagam *et al.* (1971). Thus the amount of material needed to initiate feeding behavior in *Chrysaora* polyps corresponds more closely to the amounts of amino acid one would expect to find in the seawater when nematocysts puncture the exoskeltons of crustacean prey.

Chrysaora polyps respond to increasing amounts of either brine shrimp extract or reduced glutathione with a linear increase in feeding time to a maximum value, followed by an inhibition of the response as the concentration of extract or glutathione continutes to rise, as shown in Figure 2. The differences in the concentration of glutathione necessary to cause inhibition of the feeding response cannot be explained at this time, but may be due to inherent variability between the groups of polyps used in the tests, even though all polyps were starved for one week prior to testing. However, the same general shape of all of the curves shown in Figure 2 indicates that inhibition of the feeding response occurs at higher concentrations of brine shrimp extract and reduced glutathione. In contrast, Lenhoff (1961) showed that *Hydra* respond to increasing amounts of glutathione by increasing the time spent in feeding behavior up to a steady state maximum value, indicating that stimulation of all the available receptors occurs in the presence of an excess of reagent. It appears that maximum stimulation of feeding receptors in *Chrysaora* results in less than maximum response.

In many cases, isolated tentacles exposed to amino acids and peptides reacted in a manner corresponding to tentacle behavior in the intact polyps, either by writhing or extension or both. The data suggests that at least some feeding reflex receptors are present on the tentacles and that simple reflex behavior can result from receptor stimulation. The hypothesis is further supported by data showing that glutathione at concentrations 10^{-6} M and higher was inhibitory; tentacle writhing only occured at concentrations less than 10^{-6} M reduced glutathione. This tentacle behavior is similar to that observed in whole animals. The presence of receptors on the tentacles would seem to be an evolutionary advantage, as tentacle cells are the first to contact substances emanating from prey animals punctured by tentacle nematocysts.

The minimal effective concentration for each amino acid tested varied and no correlation could be made between this concentration and the size or composition of the side groups of these amino acids. Peptides made up of two (glycylglycine) and three (reduced glutathione) amino acids induced feeding behavior. However, a peptide of 12 residues (bacitracin) and proteins such as serum albumin and trypsin did not induce feeding. Therefore it appears that a peptide which can successfully stimulate feeding behavior receptors must be of low molecular weight.

The inability of amines, aldehydes or alcohols to stimulate feeding indicates that these chemical groups alone are not responsible for activation of feeding behavior. However, the response to urea but not to acetone indicates that an amino-keto combination will initiate feeding behavior. Acetylation of the amino nitrogen groups and esterification of carboxyl groups of a number of amino demonstrates that free amino and carboxyl groups are not necessary to elicit the feeding response. Because *Chrysaora* respond to several types of amino acid and peptide-like compounds, it is possible that a number of receptor sites sensitive to a spectrum of amino acids and small peptides exist which elicit the same general pattern of feeding behavior. Minor differences in response to individual amino acids, such as control over the extent of mouth opening or tentacle writhing, may reflect this situation. Further evidence comes from the observation of the sequence of events exhibited by *Chysaora* polyps in response to the mixture of nutrients available in a dilute brine shrimp homogenate supernatant; in the lowest active concentration tentacle writhing occurred first, followed somewhat later by mouth opening, and still later by insertion of tentacles into the gut cavity. In the presence of a single agent, glutathione, all events occurred simultaneously, even at the lowest stimulatory concentration. Therefore, it is suggested that the orderly activation of each of the events in this behavioral sequence depends on the activation of receptors by more than one of the naturally occurring amino acids and peptides, even though each substance alone can activate the entire sequence if it is present in sufficient quantity, as shown in Table I. This hypothesis is supported by the work of Reimer (1971). She showed that the sea anemone, *Palythoa,* will exhibit feeding behavior when presented with filter paper soaked in relatively high concentrations of either proline or reduced glutathione (10^{-2} to 10^{-3} M). However, solutions containing both glutathione and proline were either mutually inhibitory or synergistic, depending on the proportion of each substance present. Induction of the correct food catching, mouth opening, and food swallowing sequence occurred with a particular proline-glutathione combination which was effective at a concentration approximately two orders of magnitude less than either component alone. The data suggests that different receptors in *Palythoa* respond to high concentrations of either proline or reduced glutathione indiscriminately, but respond to low concentrations of one or the other substance in a more specific manner. The end result is a modulation of the feeding response. In contrast, a clear separation of feeding behavioral events in response to chemical stimuli is shown by Lindstedt (1971) ; the sea anemone *Anthopleura* responds to the amino acid, asparagine, by contracting and bending its tentacles toward its mouth but requires stimulation by reduced glutathione in order to ingest the food once it arrives at the mouth. Regulation of feeding behavior in *Chrysaora* may be analagous to that described by Reimer (1971) for *Palythoa.*

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SUMMARY

1. Polyps of *Chrysaora quinquecirrha* exhibit characteristic feeding behavior in response to low concentrations of most common amino acids and to several small peptides.

2. Isolated tentacles also respond in characteristic fashion to amino acids and peptides. The data imply the presence of feeding reflex receptors on the tentacles.

3. Increasing concentrations of brine shrimp extract or reduced glutathione induce longer feeding response times until a maximum value is reached; further increases in extract or reduced glutathione concentration are inhibitory to the response. Thus maximum stimulation of feeding reflex receptors is inhibitory to the feeding behavior response.

4. It was not possible at this time to characterize a specific active site in amino acids or peptides which induces feeding behavior in *Chrysaora* polyps. The data suggest that *Chrysaora* possesses more than one type of receptor, and thus can interact with a number of amino acids and peptides to bring about orderly, modulated, feeding behavior.

LITERATURE CITED

Blanquet, R. S., and H. M. Lenhoff, 1968. Tyrosine enteroceptor of *Hydra*. *Science,* **159**: 633–634.

Cargo, D. G., and L. P. Schultz, 1966. Notes on the biology of the sea nettle, *Chrysaora quinquecirrha* in the Chesapeake Bay. *Chesapeake Sci.,* **7**: 95–100.

Chuin, T. T., 1930. Le cycle evolutif du scyphistomede *Chrysaora. Trav. Stat. Biol. Roscoff,* **8**: 1–79.

Florey, E., 1966. *An Introduction to General and Comparative Animal Physiology.* W. B. Saunders Co., Philadelphia, Pennsylvania, 713 pp.

Forrest, H., 1962. Lack of dependence of the feeding reaction in *Hydra* on reduced glutathione. *Biol. Bull.,* **122**: 343–361.

Fulton, C., 1963. Proline control of the feeding reaction in *Cordylophora. J. Gen. Physiol.,* **46**: 823–837.

Goreau, T. F., N. I. Goreau and C. M. Yonge, 1971. Reef corals: Autotrophs or heterotrophs? *Biol. Bull.,* **141**: 247–260.

Lenhoff, H. M., 1961. Activation of the feeding reflex in *Hydra littoralis.* Pages 203–232 in H. M. Lenhoff and W. F. Loomis, Ed., *The Biology of Hydra and Some Other Coelenterates.* University of Miami Press, Coral Gables, Florida.

Lenhoff, H. M., 1968. Behavior, hormones, and *Hydra. Science,* **161**: 434–442.

Lindstedt, J. K., L. Muscatine, and H. M. Lenhoff, 1968. Valine activation of feeding in the sea anemone, *Boloceroides. Comp. Biochem. Physiol.,* **26**: 567–572.

Lindstedt, J. K., 1971. Biphasic feeding response in a sea anemone: control by asparagine and glutathione. *Science,* **173**: 333–334.

Loeb, M., J., 1972. Strobilation in the Chesapeake Bay sea nettle, *Chrysaora quinquecirrha.* I: Effects of environmental temperature changes on strobilation and growth. *J. Exp. Zool.,* **180**: 279-292.

Loomis, W. F., 1955. Glutathione control of the specific feeding reactions of *Hydra. Ann. New York Acad. Sci.,* **62**: 209–228.

Lowry, O. H., N. J. Rosebrough, A. L. Farr and R. J. Randall, 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.,* **193**: 265–275.

Mariscal, R. N., and H. M. Lenhoff, 1968. The chemical control of feeding behavior in *Cyphastrea ocellina* and in some other Hawaiian corals. *J. Exp. Biol.,* **49**: 689–669.

Reimer, A., 1971. Chemical control of feeding behavior in *Polythoa. Comp. Biochem. Physiol.,* **40A**: 19–38.

Sober, H. A., Ed., 1968. *Handbook of Biochemistry,* Section C. The Chemical Rubber Co., Cleveland, Ohio.

Srinivasagam, R. T., J. E. G. Raymont, C. F. Moodie and J. K. B. Raymont, 1971. Biochemical studies on marine zooplankton. X. The amino acid composition of *Euphausia superba, Meganyctiphanes norvegica* and *Neomysis integer. J. Mar. Biol. Ass. U.K.,* **51**: 917–925.

Williams, R. B., 1972. Chemical control of feeding behavior in the sea anemone *Diadumene luciae* (Verrill). *Comp. Biochem. Physiol.,* **41A**: 361–371.

Reference: *Biol. Bull.*, **145**: 159–170. (August, 1973)

NEURAL CONTROL OF MIGRATION OF PROXIMAL SCREENING PIGMENT BY RETINULAR CELLS OF THE SWIMMING CRAB *CALLINECTES SAPIDUS* [1]

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Arthropods possess compound eyes which, unlike the camera type eye of vertebrates, have a number of fixed aperature lenses. Each lens serves a visual subunit (ommatidium), which is in part comprised of a number of photoreceptors (retinular cells). Each cell contributes part of the photoreceptor organelle (rhabdom) and sends an axon centrally to make synapses with visual interneurons. One of the problems associated with this type of optical system is the control of the amount of flight striking the rhabdom. A general solution to this problem has been the adaptation by which pigment granules are positioned within the ommatidium as a function of the state of light adaptation of the eye. This phenomenon of migration of screening pigment has been the subject of active research for over a century.

These screening pigments can be placed into two broad categories depending upon their position within the ommatidium (Kleinholz, 1966). The first class is located in non-visual accessory cells associated with the ommatidium and includes distal and reflecting type pigments. The second class is located within the retinular cells themselves and is termed proximal screening pigment (PSP). A persistent question has been the control of movement of these pigments; that is, do the cells containing them behave as independent effectors? The answer for the first class appears to be no, since it has been fairly well established that endocrine organs effect the movement of these accessory pigments. The majority of evidence suggests that such influences do not affect movement of pigments within the photoreceptors so that the site of control remains an open question.

Recent ultrastructural observations (Horridge and Barnard, 1965; Eguchi and Waterman, 1967; Kirschfeld and Francheschini, 1969; Butler, 1971; Kolb and Autrum, 1972) suggest that indeed retinular cells do exert direct control over the position of cytoplasmic structures as a function of the state of light adaptation, and in some cases retinomotor phenomena have been shown to cause a change in the gross morphology of the receptor cells (Walcott, 1969, 1971; Debaisieux, 1944). The similarity between these phenomena and the migration of PSP within photoreceptors suggests that the behavior of the PSP could be under neural control of these cells as well. This hypothesis is testable in ommatidia in which (a) PSP does migrate over the axial dimension of the structure and (b) in which one can deliver stimuli which differentially excite photoreceptor elements with that ommatidium.

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The sensitivity of crustacean retinular cells to the plane of polarized light is of particular interest in this regard. Ultrastructural analysis (Eguchi and Waterman, 1966), and microspectrophotometric measurements (Waterman, Fernandez and Goldsmith, 1969) have shown that the basis for this sensitivity lies in the orientation of the microvilli which comprise the rhabdom. Summarily, crustacean ommatidia contain 7 retinular cells, numbered one through seven, over the majority of their length. These cells fall into two classes based on their microvillar orientations. Cell 1 has a microvillar orientation parallel to cells 4 and 5 but occupies a position on the opposite side of the rhabdom. Cells 2 and 3 are adjacent and share a common microvillar axis with cells 6 and 7, also adjacent, located on the opposite side of the rhabdom. Furthermore, the planes of these two microvillar orientations are orthogonal and they align with the vertical axis and horizontal plane of the body. Electrophysiological measurements of polarization sensitivities of individual retinular cells confirm this relation and show that the response of a single cell to a polarized light stimulus oriented parallel to its microvillar axis is often 4 to 6 times greater, in terms of stimulus intensity, than when the same stimulus is oriented orthogonally to that axis, (Waterman and Horch, 1965; Shaw, 1966, 1969; Waterman and Fernandez, 1970; Muller, 1971; Mote, 1972). Therefore, when cells of one class are being most effectively excited, the cells of the other class are receiving stimuli which are severalfold less effective. In this paper we describe experiments in which the position of the PSP in retinular cells of the portunid crab *Callinectes sapidus* was assessed histologically after selective adaptation by polarized light. The results demonstrate that the axial migration of proximal screening pigment is a direct result of receptor excitation and is independent of excitation of other cells in the same ommatidium or eye.

MATERIALS AND METHODS

Adult specimens of *Callinectes sapidus* were obtained from food markets in the Philadelphia area shortly after capture by commercial fisheries along the coast of southern New Jersey. They were kept in 100 gallon seawater tanks under constant lighting, at 22 degrees C, and were used within 2 weeks of capture without regard for size or sex. Experiments were performed in two ways. First, intact animals were fixed to a platform, their eye stalks were immobilized with cotton wedges inserted in the orbits. Cotton wicks were placed in contact with the cornea to monitor the ERG (mass response) with standard techniques. The animals were dark adapted for at least an hour following which the evperimental eye was stimulated with flashes of light from a 6V tungsten lamp located 50 cm from the eye. The light was equipped with electromagnetic shutter and polarizing filter (Kodak KN36). The filter was oriented so as to align the e-vector with either vertical axis or horizontal plane of the eye. Flashes of light, 40 msec in duration, were delivered at the rate of 1/sec. The amplitude of the ERG response to these stimuli quickly decreased from its dark adapted value and stabilized some time later at a second value. The flash rate was then decreased until the ERG amplitude was constant at some proportion (25%, 50%, 75%) of the dark adapted value. The eye was flashed at the new rate for 20 minutes. Since the alignment of the plane of polarization of the stimulus and one set of microvillar axes in the eye could only be approximated in these experiments, a second type of experiment was performed in which proper alignment was assured by monitoring the intracellular response of individual retinular cells to polarized stimuli. Standard microelectrode techniques were employed and are described elsewhere (Bruno, Mote and Goldsmith 1973). In these experiments dark-adapted photoreceptors of isolated eye stalks were exposed by slicing the cornea with a razor blade. The base of the stalk was placed in a seawater bath while the corneal surface was in air and exposed directly to the polarized stimulus source. Maximally and minimally effective polarization orientations for the cell were located by rotating and polarizing filter and observing the response of the cell to flashes of moderate intensity. The eye was then stimulated for 20 minutes with the filter in the maximally effective orientation and the flash rate adjusted so that the amplitude of the cells response to that stimulus stabilized at a new value midway between the dark adapted maximum and minimum. Controls consisted of sham experiments using unpolarized light as the stimulus, and completely light and dark adapted animals.

Immediately following the adaptation treatment eyestalks were placed in a 5% acetic acid solution at 85° C for 30 seconds to halt further pigment movement (L. H. Kleinholz, personal communication). The stalk was then frozen by placing it in a block of dry ice for 60 seconds. The frozen stalk was then bisected in such a way that the stimulated ommatidia remained unaffected by the section. The halves were then placed in 70% ethanol overnight. Following this treatment the retina, optic ganglia and muscles could be teased from the chitinous shell of stalk and cornea, obviating the difficulties of sectioning such materials. The tissue was then inbedded in paraffin, cut into 10 micron sections, lightly stained with haematoxylin and eosin, and mounted in balsam. Photomicrographs were taken under interference contrast microscopy on a Zeiss Ultraphot II light microscope.

RESULTS

Polarization sensitivity

Retinular cells of *Callinectes* show polarization sensitivity ratios typical of other crustaceans where data is available. These cells respond to light by a depolarization of the resting membrane potential as is common among invertebrates (Fig. 1A). The amount of depolarization is dependent upon the stimulus intensity (Fig. 1B). If plane polarized light is used as a stimulus, then the amount of depolarization is also dependent on the e-vector orientation. Figure 1C shows the modulation in response amplitude as a function of that orientation. The polarizing filter was rotated in 15 degree increments and a 40 msec flash was delivered at each step. The polarization sensitivity ratio can be determined from response-energy relation (Fig. 1B) by locating the maxima and minima of Figure 1C on that curve and measuring the effective intensity of the stimulus at those orientations. The result in this case is a difference equal to about 0.6 log units, or a sensitivity ratio of about 4–1. This value is typical of values of a more extensive study on *Callinectes* (Mote, unpublished).

Pigment migration

Pigments located in non-visual accessory cells (distal and reflecting) in the photopic (apposition) type retina of *Callinectes* appear to be limited to the ex-

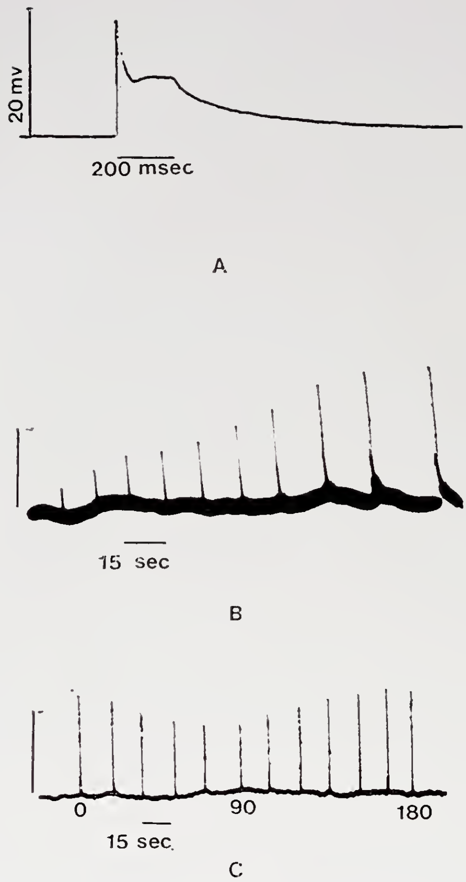

FIGURE 1. Response of a single retinular cell to (a) a 200 msec flash of light of moderate intensity; (b) response of the same cell to a series of 40 msec light flashes of increasing intensity. Each flash is twice (0.3 log units) as bright as the preceding flash and the interval between flashes is 15 seconds; (c) the response of the cell to a series of polarized stimuli of constant intensity and 40 msec duration delivered at an interval of 15 sec. During the interval the polarizing filter was rotated 15°. (The vertical calibration is the same for A, B and C.) The numerals below the trace indicate the cells' response to polarization angles of 90 and 180 degrees.

treme ends of the ommatidium and do not migrate. The distal pigment remains localized in cells around the distal end of the ommatidium where it contacts corneal structures. The reflecting pigment is localized in cells around the proximal end of the ommatidium where the retinular cell axons leave the retina.

The proximal screening pigment can be seen in histological section as a dense globular material restricted to the photoreceptor elements of each ommatidium. It appears to be localized in two masses. An external mass is found in the distal portions of the retinular cells and does not extend more centrally than the outer

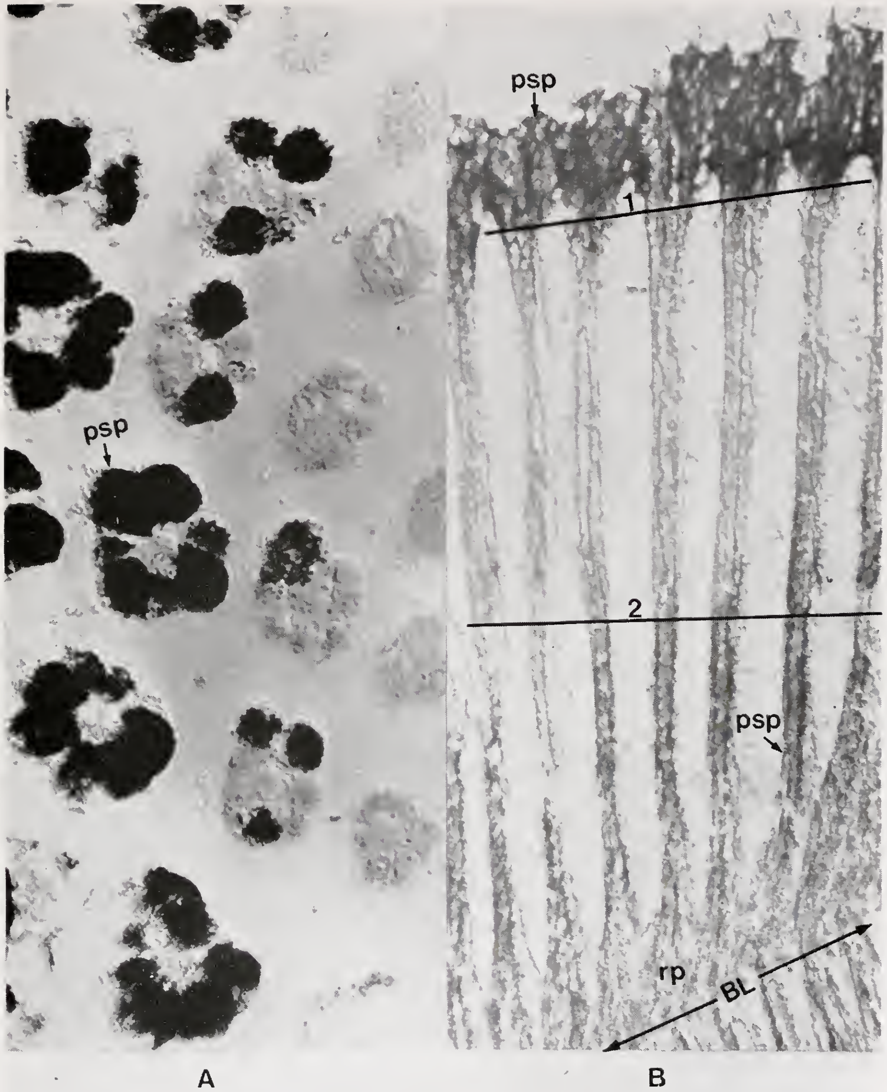

FIGURE 2. Histological section of the retina of *Callinectes sapidus* which has been selectively adapted with polarized light flashes of sufficient intensity and frequency to reduce the amplitude of the ERG response to 50% of its dark adaptd value. The cornea has been removed; (a) an oblique cross section through the retina approximately along the line marked 1 in 2B; (b) longitudinal section of several adjacent ommatidia. The thickened distal ends (top) contain the dense proximal screening pigment (PSP) which extends centrally to the region marked by line 1. The central regions of the cell also contain this material which extends distally to the area marked by line 2. Reflecting pigment (rp) is found on both sides of the basement lamina (BL) which borders the receptor layer centrally.

20% of the ommatidium (Fig. 2B). There is some suggestion of movement of this pigment mass, but if it occurs it is so restricted that it is very difficult to assess (Fig. 2A and discussion). An internal mass of the PSP is located in the proximal regions of the retinular cell and appears to migrate extensively (Fig. 2B). In animals that have been strongly light-adapted these two masses meet and the cell is completely filled with the dense screening material. In moderately light-adapted animals there is a gap between the two masses as shown in Figure 2B, and in dark-adapted animals the internal mass migrates centrally to the level of the basement lamina (Fig. 2B) and is not seen peripherally.

Longitudinal sections are useful in determining the position of the PSP within the retina, however they do not allow determination of the position of the pigment within the elements of a single ommatidium. To determine the relative distribution of pigment within the receptor cells of a single ommatidium, the ommatidium was examined in serial cross sections. Figure 3 is a photomicrograph of a cross section (approximately at the level indicated by line 2 in Fig. 2B) through a retina adapted by polarized light whose e-vector had been oriented by monitoring the intracellular response. It consists of adjacent ommatidia representing elements from three adjacent rows in the two axes of the eye and demonstrates that the PSP is not evenly distributed through all cells of each ommatidium. Furthermore it demonstrates (1) that this lack of symmetry is consistent in form among neighboring elements, (2) that it aligns with a row marking the horizontal axis of the eye (large arrow at center), and (3) that the pigment can be found on opposite sides of the rhabdom (small arrows). The form of the asymmetry is not restricted to a particular section but is also consistent along the axial dimension of the ommatidium. Figure 4 represents a series of cross sections of a single ommatidium over about 100 micra of its 300 micron length. It was taken from the same retina as that shown in Figure 3. The sections proceed proximally in 10 micron steps except where sections omitted because of redundancy (see caption). The PSP first appears along the rhabdom as a thin layer which extends for about 50 microns (Fig. 4A–D), at which point it appears around 2 adjacent reticular cell nuclei and on the opposite side of the rhabdom (Fig. 4E). In Figure 4F the pigment has surrounded the rhabdom but is predominantly located in the elements on opposite sides, and finally (Fig. 4E–I) is distributed equally in all elements. Figure 5 represents a series taken from a retina treated identically to that depicted in Figures 3 and 4 but omits alternate sections. It demonstrates the essential features [*i.e.*, (a) appearance of pigment first along the rhabdom, and (b) then around the nucleus, (c) in cells on opposite sides of the rhabdom, and (d) finally in all elements] that are similar in all serial sections we were able to construct. Thus it appears that the PSP can be localized in different axial positions in different retinular cells of a given ommatidium that has been selectively adapted with polarized light. Furthermore this distribution appears to be consistent among neighboring ommatidia in a given region of the eye. In 15 selective adaptation experiments, results of histological examination suggested uneven pigment distribution while in 5 sham control experiments, or in fully light or dark adapted eyes they did not.

In experiments on intact eyes, the orientation of the adapting filter with respect to the major axes of the eye was only approximated and the process of

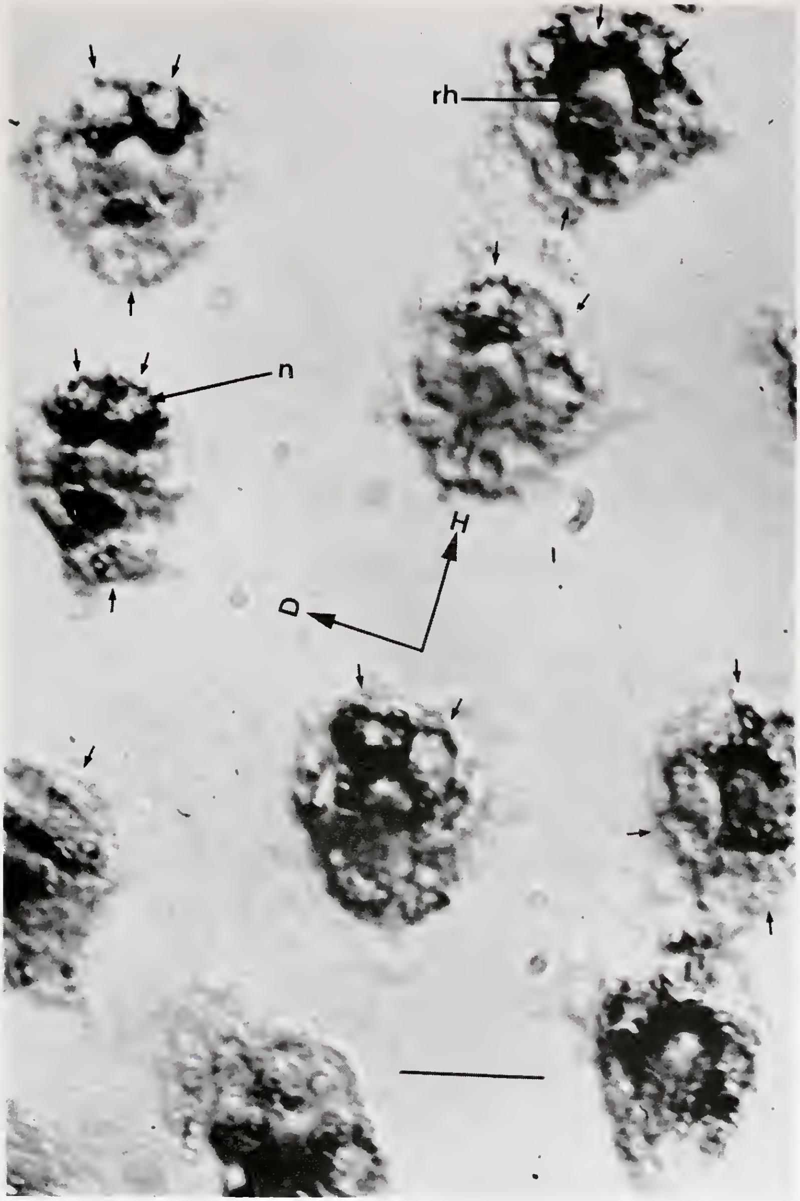

FIGURE 3. Cross section through a retina selectively adapted with flashes of polarized light whose e-vector orientation had been determined by monitoring the intracellularly recorded receptor potential. The plane of section coincides approximately with line 2 of Figure 2B. The proximal screening pigment appears in various elements of each ommatidium and is marked by the small arrows. The large arrows note the dorsoventral (D) axis and horizontal (H) axis of the eye. Each ommatidium shows a pigment free central area, the rhabdom (rh), and pigment free peripheral areas, the retinular cell nuclei (n). Scale bar indicates 20 microns.

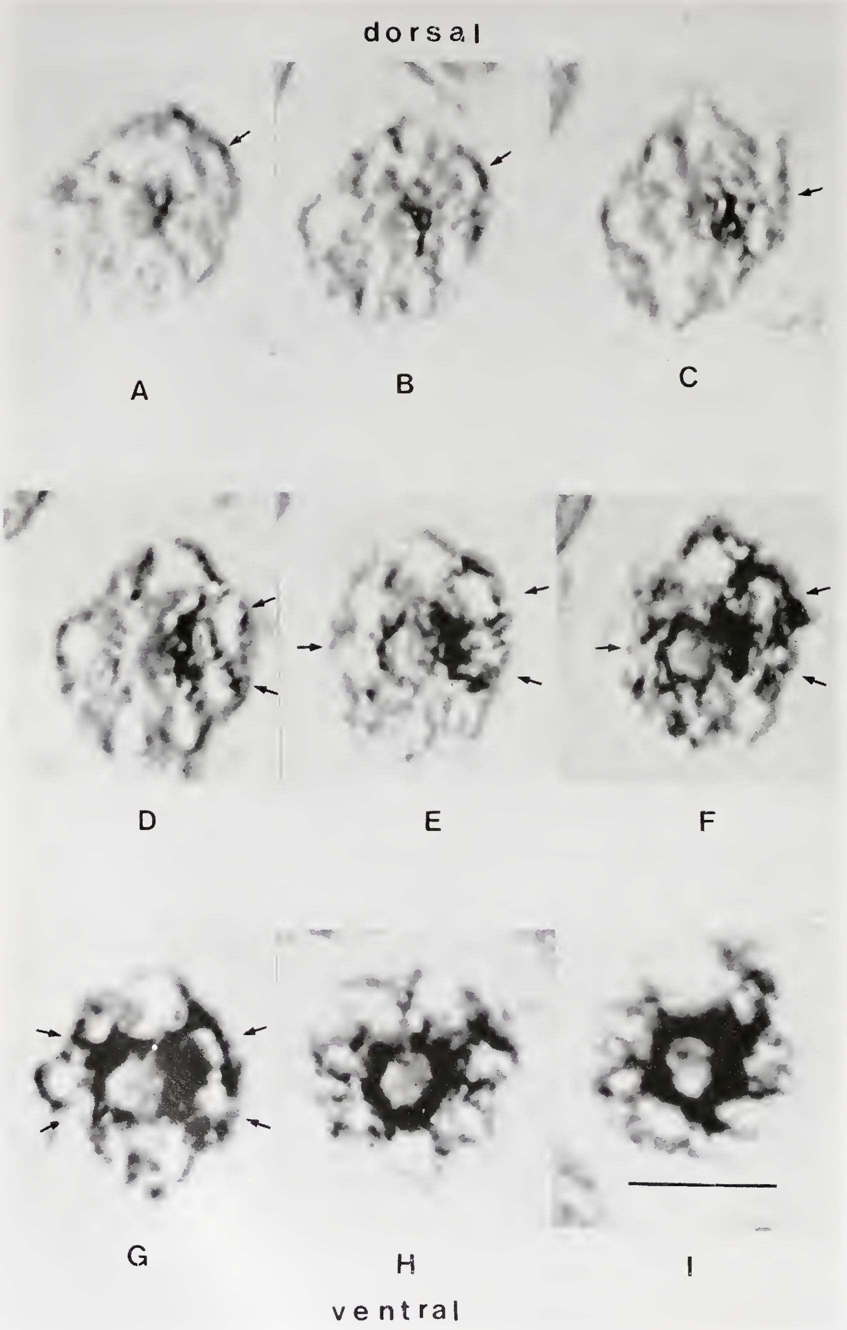

FIGURE 4. Serial cross sections through a single ommatidium from a retina selectively adapted with flashes of polarized light whose e-vector had been oriented by monitoring the intracellular response. The first section (A) was made approximately 100 microns from the

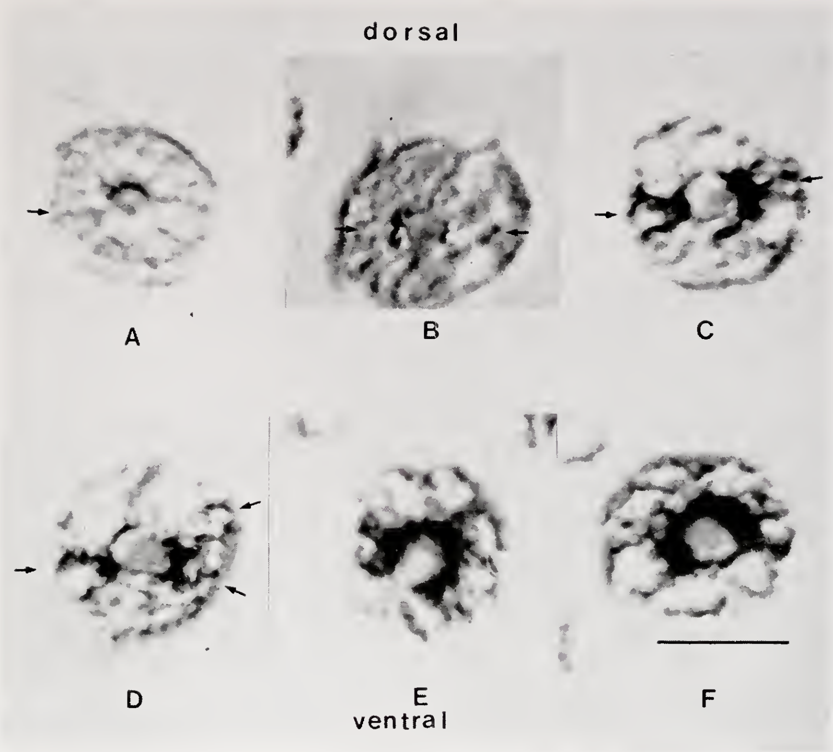

FIGURE 5. Serial cross sections through an ommatidium in a retina treated as in Figure 4. The first section (A) begins at about the same depth as in Figure 4, but only alternate sections are shown. Thus the axial extent of the series is about 100 microns. Small arrows in sections B–D indicate where the proximal pigment can be observed. Scale bar indicates 20 microns.

preparation for histological section produced major uncertainties regarding the orientation of tissue in the paraffin block. We can, however, estimate the relation of pigment position to the major axes in experiments where the filter was positioned by monitoring the intracellular response to polarized stimuli because in this type of experiment the retina was sliced in a plane which was approximately parallel to the horizontal axis, the cut edge then providing a landmark in each section. These estimations are included in Figures 3, 4 and 5. It must be noted that in such experiments the filter position was appropriate for a single cell and its relation to the whole eye can only be inferred. Furthermore, we cannot preclude distortions caused by electrode impalement or histological preparation.

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distal end of the ommatidium (roughly midway between lines 1 and 2 of Fig. 2B) and proceed centrally at 10 micron intervals. One section has been omitted between C and D, and between G and H so that the axial extent of the series is about 110 microns. Small arrows in sections A–C indicate regions of the ommatidium where the proximal screening pigment can be observed. In sections H and I the pigment clearly surrounds the entire rhabdom. Scale bar indicates 20 microns.

Given these reservations, we feel that the ommatidial diameter along which the PSP is distributed aligns reasonably well with the estimated horizontal axis of the eye stalk in Figures 3, 4 and 5.

DISCUSSION

The only motile pigment found in the retina of *Callinectes sapidus* was that which was localized in the photoreceptor elements and can be classified as proximal screening pigment (PSP). Our examinations revealed that it is found in two masses, external and internal. The external mass, if mobile at all, remains in the peripheral regions of the ommatidium. Sections through this region of the retina occasionally suggested uneven distribution of this material (Fig. 2A), however, the restricted region of the ommatidium in which this material could be observed makes it difficult to eliminate the possibility of artifact or make any clear statement about its mobile properties. The internal mass of PSP, on the other hand, can clearly be found in different positions within the retinular cells of the same ommatidium when the retina had been adapted with polarized light. Intracellular measurements of polarization sensitivity ratios in these cells yielded values of between 4 and 6 to 1. This is the same as saying that a given polarized stimulus, if properly oriented, excites one set of receptors (*e.g.,* 2, 3, 6 and 7) as though it were a light 4 to 6 times brighter (or dimmer) than the other set (*e.g.,* 1, 4 and 5). If the screening pigment were to move independently in each set as a function of the stimulus intensity, then one would expect to find the pigment in different positions in each set. Our results suggest that this is the case.

The difficulties inherent in properly orienting and sectioning experimental eyes raise the possibility that such results are artifactual. The ultrastructure of the ommatidium as described above poses certain restrictions upon the types of uneven distributions one would expect to find if the pigment movement were in response to the adapting stimulus. First, the distribution should be similar in a number of adjacent ommatidia since their microvillar axes are in register along each row and a stimulus properly aligned for one would be properly aligned for others as well. Figure 3 demonstrates that this requirement is fulfilled. The elements depicted there show pigment distributions comparable to each other and these distributions align with the axis of the row. Secondly, since retinular cells on opposite sides of the rhabdom are receiving stimuli which are equally effective (see above) one would expect the pigment to appear along a diameter of the ommatidium. Figures 3, 4 and 5 demonstrate that this requirement is fulfilled. We imposed the further requirement that we be able to follow a single ommatidium in serial sections and observe the appearance of the PSP at its distal limit. This is a severe requirement for several reasons. First, the ommatidial axes are not parallel so that the chance that a given plane of section will cut the same element in cross section over a hundred microns is small. Secondly, the theoretical treatment of Snyder and Pask (1972), based on the rhabdom of the honey bee, suggests that the polarization sensitivity will fall off sharply as the position of the stimulus deviates from the optical axis of the ommatidium. This means that in our experiments a relatively small number of ommatidia would be receiving the appropriate stimulus from a localized source 50 cm away, and the difficulty of obtaining them in cross section is compounded.

We feel that the serial sections depicted in Figures 4 and 5 fill this requirement. They demonstrate that the observed distributions persist over some distance and that the transition from asymmetrical to symmetrical distribution is gradual. We consider it unlikely that an artifact created by the plane of section or tissue damage during sectioning, could result in such observations. This, coupled with the agreement between our results and the predictions from ultrastructural analysis and electrophysiology, lead us to conclude that the hypcthesis that these photoreceptors exert direct action on the pigment material within them is correct. Unfortunately our experiments do not enable us to comment on the mechanism of movement or even quantitatively describe it.

One must use caution, however, in generalizing these results to other species. Kleinholz (1961) points out that the presence of the different types of screening pigments and their motility is variable in different species. On the other hand, the preponderance of evidence supports hormonal control of distal and reflecting pigment migration where they have been observed, while such a mechanism does not appear to affect migration of the PSP. In this light, our experiments suggesting neural control of PSP migration tempt the speculation that such a mechanism is a general property of arthropod photoreceptor cells. In addition, these experiments and their results serve as further evidence of the functional independence of retinular cells within each ommatidium of the arthropod compound eye even though these cells share a common rhabdom.

We wish to thank Professor Lewis Kleinholz for his helpful advice on histological techniques.

SUMMARY

1. The position of proximal screening pigment (PSP) located in retinular cells of the compound eye of the crab *Callinectes sapidus* was assessed histologically after selective adaptation with plane polarized light.

2. The results showed the pigment position to be different within the cells of a single ommatidium.

3. The pigment position was similar in cells of that ommatidium which shared a common microvillar axis and different from those that did not.

4. The pattern of pigment distribution was similar in several adjacent ommatidia.

5. Serial sections showed that the pigment in cells sharing a common microvillar axis extended more distally than those that did not after a polarized adapting stimulus.

6. These results support the hypothesis that the migration of PSP is under neural control of the retinular cell where it is found and independent of activity in other cells in the same ommatidium or eye.

LITERATURE CITED

BRUNO, M., M. I. MOTE AND T. H. GOLDSMITH, 1973. Spectral absorption and sensitivity measurements in single ommatidia of the green crab, *Carcinus*. *J. Comp. Physiol.*, **82** (2): 151–164.

Butler, R., 1971. The identification and mapping of spectral types in the retina of *Periplaneta americana*. *Z. Vergl. Physiol.*, **72**: 67–80.

Debaisieux, P., 1944. Les yeux des crustaces. *La Cellule*, **50**: 9–122.

Eguchi, E., and T. H. Waterman, 1966. Fine structure patterns in crustacean rhabdoms. Pages 105–124 in C. G. Bernhard, Ed., *The Functional Organization of the Compound Eye*. Pergammon Press, New York.

Eguchi, E., and T. H. Waterman, 1967. Changes in retinal fine structure induced in the crab *Libinia* by light and dark adaptation. *Z. Zellforsch*, **79**: 209–229.

Horridge, G. A., and P. B. T. Barnard, 1965. Movement of palisade in locust retinular cells when illuminated. *Quart. J. Microscop. Sci.*, **106**: 131–135.

Kirschfeld, K., and N. Franceschini, 1969. Ein mechanismus zur steurung des lichtfluss in den rhabdomeren des komplex auges von *Musca*. *Kybernetik*, **6**: 13–22.

Kleinholz, L. H., 1961. Pigment effecters. Pages 133–169 in T. H. Waterman Ed., *The Physiology of Crustacea Vol. II*. Academic Press, New York.

Kleinholz, L. H., 1966. Hormonal regulation of retinal pigment migration in crustaceans. Pages 89–101 in C. G. Bernhard, Ed., *The Functional Organization of the Compound Eye*. Pergammon Press, New York.

Kolb, G., and H. Autrum, 1972. Die feinstruktur im auge der biene bei hell und dunkeladaptation. *J. Comp. Physiol.*, **77**: 113–125.

Mote, M. I., 1972. Polarization sensitivity ratios of retinular cells in the crabs *Carcinus* and *Callinectes* under conditions of selective adaptation and dim stimuli. *Biol. Bull.*, **143**(2): 471.

Muller, K., 1971. An electrophysiological investigation of retinular cells in the compound eye of the crayfish, *Procambarus clarkii*. *PhD. thesis, Massachusetts Institute of Technology*, Cambridge, Massachusetts.

Shaw, S. R., 1966. Polarized light response from crab retinula cells. *Nature*, **211**: 92.

Shaw, S. R., 1969. Sense cell structure and interspecies comparisons in arthropod compound eyes. *Vis. Res.*, **9**: 1031–1040.

Snyder, A. W., and C. Pask, 1972. Detection of polarization and direction by the bee rhabdom. *J. Comp. Physiol.*, **78**: 346–355.

Walcott, B., 1969. Movement of retinula cells in insect compound eyes on light adaptation. *Nature*, **223**: 971–972.

Walcott, B., 1971. Cell movement on light adaptation in the retina of *Lethocerus* (Belostomidae, Hemiptera), *Z. Vergl. Physiol.*, **74**: 1–16.

Waterman, T. H. and H. R. Fernandez, 1970. *E*-vector and wavelength discrimination by retinular cells of the crayfish *Procambarus*. *Z. Vergl. Physiol.*, **68**: 154–174.

Waterman, T. H., H. R. Fernandez and T. H. Goldsmith, 1969. Dichroism of photosensitive pigment in rhabdoms of the crayfish *Oroconectes*. *J. Gen. Physiol.*, **54**(3): 415–432.

Waterman, T. H. and K. W. Horch, 1965. Mechanisms of polarized light perception. *Science*, **154**: 467.

Reference: *Biol. Bull.*, **145**: 171–179. (August, 1973)

EFFECTS OF β-ECDYSONE ON MOLT-LINKED DIFFERENTIATION *IN VITRO*

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The occurrence of morphogenic activity in cultured tissues and organs of insects has been reported by a number of researchers (see Marks, 1970). However, the results of these studies have not always been easy to interpret. In their work with *Drosophila,* both Demal (1961) and Schneider (1966) pointed out that the stage of development of the tissue donor greatly affected the amount of morphogenesis that occurred, and Mandaron (1971) showed that α-ecdysone induced differentiation in explanted imaginal disks. Further, Oberlander and Fulco (1967) and Oberlander (1969) showed that the presence of ecdysone analogs in the culture medium enhanced morphogenic development in wing imaginal disks of *Galleria.*

Regenerate appendages in cockroaches may be formed during any instar, including those that occur within the egg (Bullière, Bullière, and Sengel, 1969). In nymphal cockroaches, a regenerate complete with setae and spines can be formed within a single instar only if the leg is removed early in the instar. If the leg is removed late in the instar, only an undifferentiated papilla is formed (O'Farrell and Stock, 1953).

Marks and Reinecke (1965) showed that incubation of very young leg regenerates of cockroaches with prothoracic glands from older insects resulted in a loss of organotypic activity and atrophy of the migrating cells. Since then, Bullière and Bullière (1970) convincingly demonstrated that the initiation of leg regeneration can be inhibited by treatment with molting hormone *in vitro.*

Marks and Leopold (1971) showed that treatment of mature (25-day-old) leg regenerates with β-ecdysone induced differentiation of tormogen and trichogen cells from the epidermis and that the frequency with which seta formation occurred was related to the age *in vivo* of the explant. Thus, *in vitro* the same dose of ecdysone that inhibits the initiation of regeneration enhances the development of a mature regenerate. Clearly, then, the presence of molting hormone, either exogenous or endogenous, has a pronounced effect on the course of development in cultured tissues and organs, and the effect is dependent on the age *in vivo* of the target tissue.

A second process that is related to development and can be initiated by the presence of molting hormone *in vitro* is cuticle deposition (Agui, Yagi and Fukaya, 1969; Marks and Leopold, 1970; Oberlander and Tomblin, 1972). The deposition of cuticle by cockroach leg regenerates *in vitro* also depends to some extent on the age of the tissue *in vivo* (Marks and Leopold, 1971), but far more so on the amount of exogenous molting hormone and the duration of exposure to it (Marks, 1972a).

Thus, morphogenesis in cockroach leg regeneration depends on the age *in vivo* of the explant and is linked with the process of cuticle deposition and molting. To see whether we could define more precisely the relationship between these two processes, we undertook an *in vitro* study of the effects of the molting hormone β-ecdysone on seta formation and cuticle deposition in leg regenerates of cockroaches of different ages.

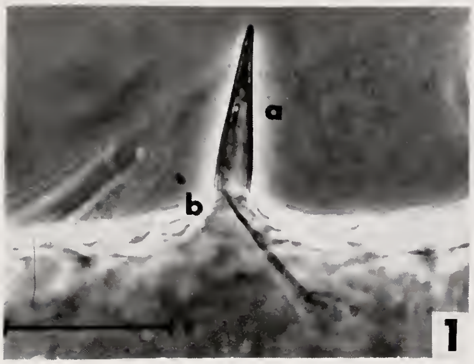
METHODS

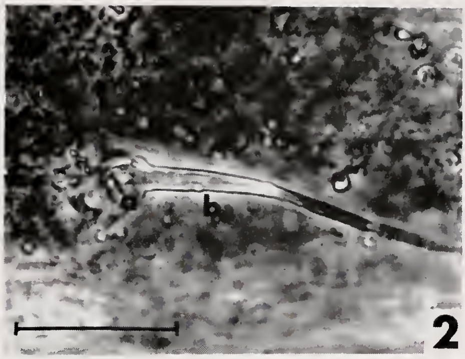
Newly molted late-instar nymphs of the cockroach, *Leucophaea maderae* (F.), were removed from the colony and held for 24 hr. Then the mesothoracic legs were removed at the coxo-trochanteral joint, and the insects were held an additional 10–30 days in paper cups and fed dog chow and water while leg regenerates developed. The insects were then surface-sterilized, and the leg regenerates were dissected from the coxal stump. The explants were placed under dialysis strips in multipurpose tissue chambers (Rose, 1954), and the chambers were filled with M20 culture medium (2 ml) supplemented with 7.5% fetal calf serum (fcs) (Marks, 1973). Crystalline β-ecdysone dissolved in water (1 μg/μl) was injected into the chambers with a microsyringe. In the first set of experiments, doses of 1, 5 and 10 μg/ml were given 24 hr after explanation and allowed to remain in the chambers for 5 days. In a second set of experiments, β-ecdysone (2.5 μg/ml) was injected into the chambers along with puromycin (0.5 μg/ml) and allowed to remain for 3 days. After exposure, all chambers were rinsed twice, refilled with culture medium and incubated at 27° C. The explants were examined by phase contrast microscopy after 10 days of incubation and again after 14 days. The criteria for the presence of cuticle were those of Marks (1972a), and each specimen was examined carefully for identifiable setae. Since many kinds of aberrant surface sculpture appeared on the explants when the cuticle was deposited, a structure was scored as a seta only if the trichogen cell or the shaft of the seta could be identified within the tormogen cell or socket. The outline of the explant was scanned for setae in profile (Fig. 1); if none was found, a detailed search of the surface of the explant was made at higher power (Fig. 2). The frequencies of occurrence of seta formation and cuticle deposition were measured for a minimum of 10 specimens for each dosage. The data were transformed—frequencies to probits and dosages to logs—to straighten the otherwise sigmoid curve, and a standard regression analysis was run to permit extrapolation.

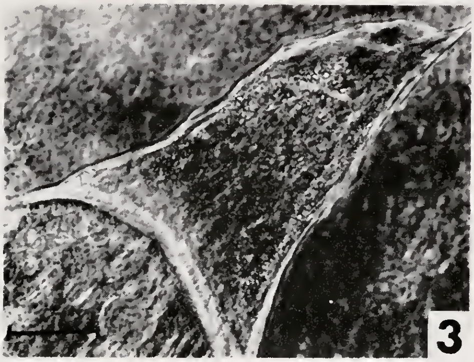
RESULTS

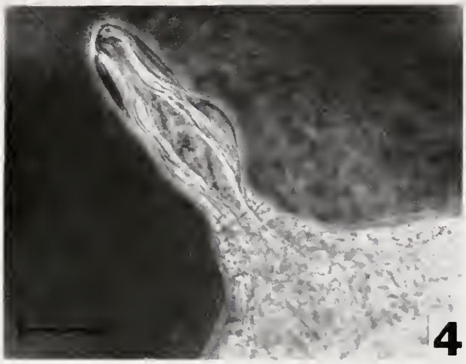
Numerous types of sculpture were found on the cuticular surface of treated explants; these included fixed spines, pegs, unidentified ring-shaped ridges that may have represented aberrant tormogen cells, and scale-like sculpture (Figs. 3–6). Many of the pits and fixed spines occurred regularly when older specimens were given large doses of the hormones, but other structures (for example, the scale-like sculpture) were observed occasionally and then only on small portions of the explant.

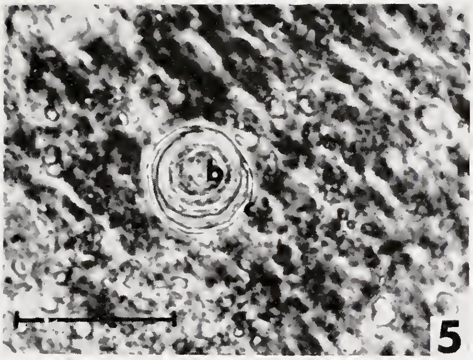
In young tissues up to 20 days *in vivo,* as many as 40% did not respond to molting hormone, perhaps because of damage during explantation. To eliminate these unresponsive explants from consideration, we divided the frequency of seta formation by the frequency of cuticle deposition to obtain a ratio of seta to











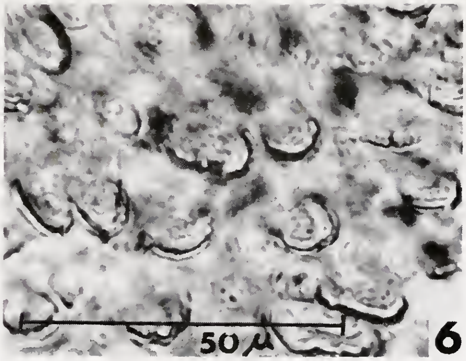


FIGURE 1. Seta shown in profile. Shaft (trichogen *a*) is seated in the socket (tormogen *b*); 26 days *in vivo;* 24 days *in vitro.*

FIGURE 2. Seta shown in surface view. Sclerotization of tormogen (a) and trichogen (b) is incomplete; 26 days *in vivo;* 24 days *in vitro.*

FIGURE 3. Multicellular spine formed on surface of explant is well-sclerotized; 26 days *in vivo;* 24 days *in vitro.*

FIGURE 4. Cuticular peg is of multicellular origin; 24 days *in vivo;* 30 days *in vitro.*

FIGURE 5. Circular pit contains ring-shaped cell (b), which may represent aberrant tormogen cell, within a circular ridge of cuticle (c); 26 days *in vivo;* 28 days *in vitro.*

FIGURE 6. Noncellular, scale-like, cuticular processes appear in patches and resemble sculpture found on mature legs; 26 days *in vivo;* 22 days *in vitro.*

cuticle. This ratio provided us with a measure of the frequency with which tissues that produced cuticle also produced setae (Table I).

In the 10- and 15-day age groups there were no significant differences in the frequency of seta formation or of cuticle deposition among either dose levels or age groups. The mean frequency for cuticle deposition was 61.2% and for seta formation, it was 5.7%. The resultant seta cuticle ratio varied from 0 to 12. The difference in frequency between the 15- and 20-day age groups is significant at the 95% level of confidence for both cuticle deposition and seta formation. The frequency of cuticle deposition was approximately 95% for all dose levels for all leg regenerates older than 20 days. The mean frequency of seta formation increased fourfold between 15 and 20 days and doubled again between 20 and 30 days, as did the seta/cuticle ratio. These differences were significant at the 95% level of confidence. Within the 30-day age group, there was also a significant difference between the 1 and 10 µg/ml dose levels.

TABLE I

The effect of age of explant in vivo *and dose of β-ecdysone on cuticle deposition and seta formation by cockroach leg regenerates* in vitro

Age (days) of explant *in vivo*	Dose* (5-day exposure)		Number specimens	Percentage of response of		
	µg/ml	µgd		Cuticle	Seta	Seta/cuticle
10	1	5	14	57.1	0	0
	5	25	17	47.0	5.9	12
	10	50	14	64.3	7.1	11
15	1	5	15	66.7	0	0
	5	25	15	60.0	0	0
	10	50	14	71.4	7.1	10
20	1	5	13	100.0	38.5	38
	5	25	15	93.3	26.7	29
	10	50	13	92.3	30.8	33
25	1	5	14	85.7	35.0	41
	5	25	16	96.2	50.0	52
	10	50	15	96.7	53.3	55
30	1	5	12	100.0	41.8	42
	5	25	12	91.6	75.0	81
	10	50	13	100.0	76.9	77

* In no case did leg regenerates younger than 30 days *in vivo* produce cuticle or setae unless treated with molting hormone *in vitro*.

The effects of a wider range of doses on 25-day-old leg regenerates are shown in Figure 7. From these results, we can see that when the slopes for seta formation and cuticle deposition are extrapolated, they have a common origin at the 2% level of response (0.35 µgd), but that the rate of increase in response at a given dose is fourfold greater for cuticle deposition than for seta formation. The frequency of cuticle deposition increased with an increase in dose up to 2 µgd. Above this dose, 99% responses occasionally occurred, but it happened more frequently that one or two specimens failed to respond at all, even at high levels. Since this was apparently a random occurrence, we used the mean response for doses above 3 μgd as the practical maximum response. The response of seta formation rose more slowly than that of cuticle deposition, but even at the maximum response level of 18 μgd, only 50% of the specimens produced setae.

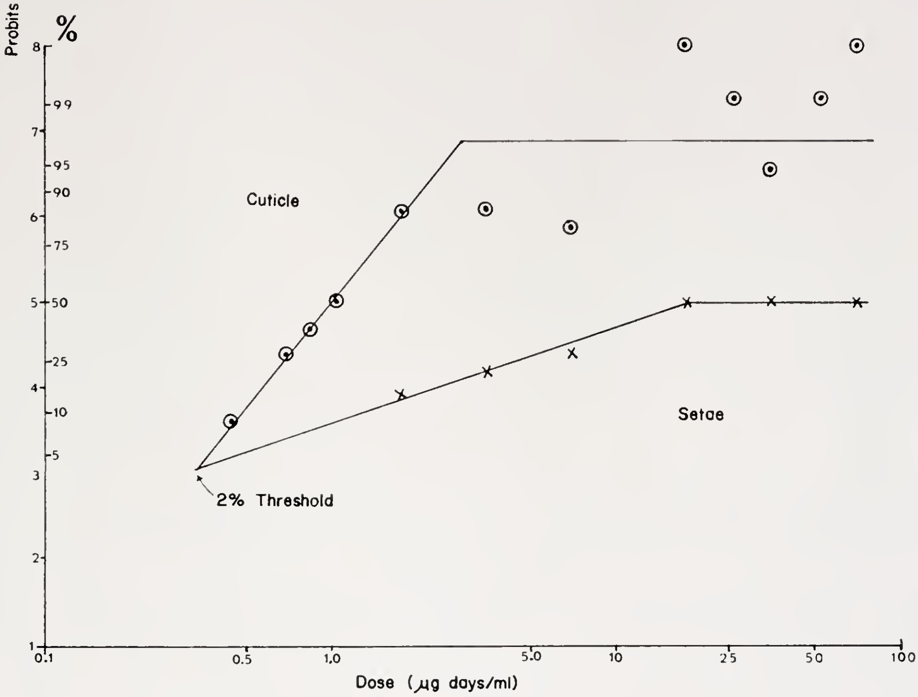


FIGURE 7. Effect of β-ecdysone on cuticle deposition and seta formation in 25-day-old cockroach leg regenerates; regression for cuticle deposition (⊙) up to 5 days is $y = 0.89 + 4.10$ (10×); $r = 0.99$ and regression for seta formation (×) up to 15 days is $y = 2.56 + 1.06$ (10× ; $r = 0.99$.

It was apparent from our earlier work that the age of the specimen *in vivo* was also a critical factor in determining the response (Marks and Leopold, 1971). Therefore, we ran an additional series of experiments in which the dose was held constant and the age of the specimen *in vivo* was varied from 10 to 30 days. The dose of hormone was kept constant at 25 μgd, and the frequencies of cuticle deposition and seta formation were plotted against the age of the leg regenerates *in vivo*. Under these conditions, the frequency of cuticle deposition rose rapidly from 10 to 25 days. However, the frequency of seta formation rose only after a delay of 15 days (Fig. 8). In both cases, the coefficient of correlation was sufficiently high (better than 0.90) to permit extrapolation of the lines to the 99.9% response level. When this was done, the two slopes met at a point corresponding to an age of 55 days *in vivo*. These data fit in well with those from our earlier study in which

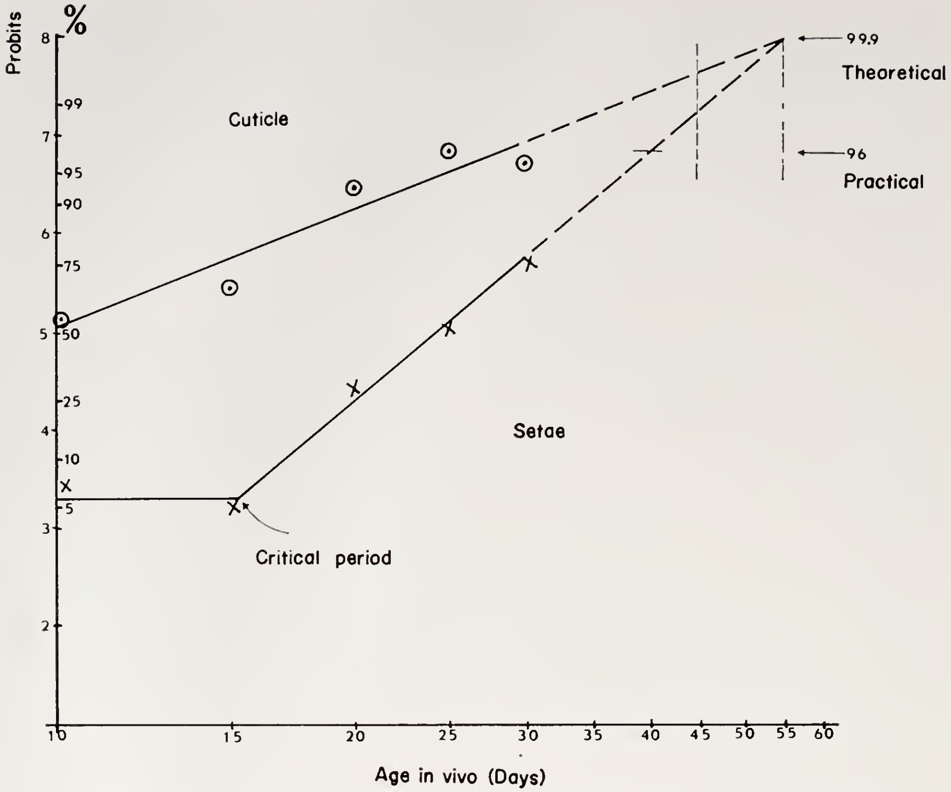


FIGURE 8. Effect of age in vivo on the induction by β-ecdysone (25 μgd) of cuticle deposition and seta formation in cockroach leg regenerates; regression for cuticle deposition (⊙) is $y = 1.13 + 3.92x$; $r = 0.92$ and regression for seta formation (×) from 15 to 30 days is $y = 8.15x - 6.31$; $r = 0.99$.

we found that 75% of 30-day-old regenerates that were induced to molt also bore setae; that 54% of 35-day-old regenerates developed without exogenous hormone, and that by 45 days, the new cuticle with setae was present at the time of explantation (Marks and Leopold, 1971). The average stadium was 52 days with a range of 45–60. Thus the slopes for cuticle deposition and seta formation met at an

TABLE II

The effect of puromycin on the induction by β-ecdysone of cuticle deposition and seta formation in vitro

Dose (μg/ml)		Days *in vitro*	Number specimens	Percentage response	
β-ecdysone	Puromycin			Cuticle	Seta
None	None	3	15	0	0
2.5	None	3	15	96	33
2.5	0.5	3	15	93	0

age well within the normal range for molting and demonstrated graphically the coordination between the processes of morphogenesis and cuticle formation.

When puromycin was added to cultured 25-day leg regenerates along with 2.5 μg/ml of β-ecdysone, cuticle was produced by more than 90% of the specimens, but no seta formation occurred. In comparison, 33% of the control specimens produced setae. Thus, a dose of puromycin sufficient to halt seta formation entirely had no apparent effect on the frequency of cuticle deposition (Table II).

DISCUSSION

All cuticular structures formed on the cultured leg regenerates are the results of morphogenic processes, but only setae could be readily identified as unmistakable cases of differentiation since it was apparent from our time-lapse photographs of seta formation *in vitro* that the tormogen and trichogen cells arise directly from cuboidal epidermal cells. This occurred several hours before the first evidences of cuticle deposition appeared (Marks, 1972b).

It was also apparent from our time-dose studies that these processes depend on the age of the tissue *in vivo* and on the dose of β-ecdysone received. If we use the frequency of response to a given level of hormone as a measure of the readiness of the tissue to respond, then the frequency of cuticle deposition represents molt readiness and that of seta formation represents differentiation readiness. However, the degree of readiness or competence to respond to doses of exogenous hormone depends on the age of the tissue *in vivo*.

Ohtaki *et al.* (1968), in their work with larvae of *Sarcophaga,* found that at no time during the last larval stadium did the titer of ecdysone reach the level required to induce molting, but at no time was it entirely absent. Nevertheless, when the larvae reached a given age, pupation took place. They explained this by postulating that the gradual accumulation of covert "hormone-initiated events" (hie) over a period of time eventually reached a number sufficient to trigger pupation. Marks (1972a) reported a similar situation with cockroach leg regenerates in which repetition of subthreshold doses of β-ecdysone *in vitro* eventually triggered cuticle deposition. If we assume that hie accumulate throughout the stadium in response to a low but constant titer of hormone *in vivo,* then the competence of tissue to respond reflects the number of hie present at any given time.

In the present study, when mature leg regenerates were treated with β-ecdysone *in vitro,* the number of hie accumulated *in vivo* was augmented artificially, and cuticle deposition and the differentiation of setae occurred within a few days. However, these two processes—molting and differentiation—responded differently to the augmented accumulation of hie. As the hormone dosage *in vitro* was increased, the frequency of cuticle deposition in 25-day-old regenerates increased rapidly to 96%, but seta formation rose only to an age-limited maximum. When the dose was held constant and the age *in vivo* was increased, the age-limited maximum increased progressively. Apparently, the initiation of cuticle deposition depends almost entirely on the accumulation of hie, but seta formation involves an additional age-dependent factor. The existence of this additional factor was demonstrated by the addition of puromycin, which effectively blocked seta formation but did not affect cuticle deposition. Although seta formation is dependent on the same β-ecdysone trigger, it also requires simultaneous protein synthesis.

The changes in the relative sensitivities of seta formation and cuticle deposition to a given number of hie provide the coordination that assures simultaneous completion of these two processes. Thus, during the first 15 days *in vitro,* the leg regenerates exhibited a high degree of molt readiness but a low degree of differentiation readiness, and the induction of molting by the addition of exogenous hormone during this critical period resulted in cuticle deposition without differentiation. A comparable situation *in vivo* resulted in the formation of a sclerotized papilla when a leg was removed late in the stadium (see O'Farrell and Stock, 1953).

In his studies of diapausing pupae of *Samia cynthia* (Drury), Williams (1968) found that the administration of large doses of β-ecdysone and other compounds with molting hormone activity before the onset of adult development caused an acceleration of the events related to metamorphosis. The doses also caused numerous developmental abnormalities that included underdeveloped legs and genitalia, patches of pupal cuticle, and the loss of normal cuticular ornamentation. Such abnormalities probably occurred because of the partial failure of the differentiation processes that normally accompany adult development. When such doses were given 60 hr after the onset of adult development, the resulting moths were normal. Socha and Senhal (1972) obtained similar results in their experiments with *Tenebrio.* Also, Judy and Gilbert (1970) reported that treatment of hindgut from pupae of *Manduca* sexta (L.) with β-ecdysone early in the instar caused cuticle to be deposited on the rectal pads before development was complete. Apparently, the premature deposition of cuticle with accompanying incomplete differentiation of the epidermal cells is a common response of tissues when molting hormone is applied early in the development cycle.

When the results of the present experiments *in vitro* are compared with the *in vivo* experiments of Williams (1968) a general agreement is apparent. The speeding up of developmental processes in response to exogenous hormone applied late in the cycle and the loss of setae on the cuticle in response to exogenous hormone applied early in the cycle are common to both experiments. The suggestion that cuticle deposition stops further differentiation of epidermal structures is supported by both the time-dose and time-lapse studies.

The present study thus confirms the hypothesis of Williams (1968) and extends it to include regeneration in paurometabolous insects. It also provides some insight into the way in which the processes leading to cuticle deposition interact with the processes leading to tissue competence. In addition, our results suggest that the use of *in vitro* techniques may make it possible to separate the process of seta formation into its various component parts. These can then be studied in isolation. Such studies may eventually lead us to a better understanding of the nature of tissue competence.

I express my appreciation for the assistance rendered by T. S. Adams of this laboratory in the preparation of the statistical analyses used in this study and for his numerous suggestions throughout the preparation of the manuscript.

SUMMARY

A study of the effects of β-ecdysone on the initiation of cuticle deposition and seta formation by cockroach leg regenerates *in vitro* showed that both processes are

ecdysone-dependent and are initiated by the same threshold dose, but the responses differ qualitatively and quantitatively. The initiation of cuticle deposition depends primarily on the accumulation of hormone-initiated events by the target tissue. The initiation of seta formation has an additional requirement for simultaneous protein synthesis.

LITERATURE CITED

AGUI, N., S. YAGI AND M. FUKAYA, 1969. Induction of moulting of cultivated integument from a diapausing rice stem borer larva in the presence of ecdysterone. *Appl. Entomol. Zool.*, **4**: 156–157.

BULLIÈRE, D., F. BULLIÈRE AND SENGEL, 1969. Regeneration du tarse chez l'embryon de *Blabera craniifer* (Insecte, Dictyoptere) en culture *in vitro*. *C. R. Acad. Sci. Paris*, **269**: 355–357.

BULLIÈRE, D., AND F. BULLIÈRE, 1970. Regeneration et facteures endocriniens chez les insectes. *C. R. Acad. Sci. Paris*, **271**: 353–356.

DEMAL, J., 1961. Problèmes concernant la morphogenèse *in vitro* chez les insectes. *Bull. Soc. Zool. Fr.*, **86**: 522–533.

JUDY, K., AND L. GILBERT, 1970. Effect of juvenile hormone and molting hormone on rectal pad development in *Hyalophora cecropia* (L.). *J. Morphol.*, **131**: 301–314.

MANDARON, P., 1971. Sur le mechanisme de l'evagination des disque imaginaux chez la *Drosophile*. *Develop. Biol.*, **25**: 581–605.

MARKS, E. P., 1970. The action of hormones in insect cell and organ cultures. *Gen. Comp. Endocrinol.*, **15**: 289–302.

MARKS, E. P., 1972a. Effects of ecdysterone on the deposition of cockroach cuticle *in vitro* *Biol. Bull.*, **142**: 293–301.

MARKS, E. P., 1972b. The induction of molting in insect organ cultures. *Proc. III Int. Colloq. Invertebrate Tissue Culture, Smolenice, Czech.*, **1971**: 221–232.

MARKS, E. P., 1973. Cockroach and grasshopper tissue. Pages 153–156 in P. F. Kruse and M. K. Patterson, Eds., *Methods and Applications of Tissue Culture.* Academic Press, New York.

MARKS, E. P., AND R. A. LEOPOLD, 1970. Cockroach leg regeneration: the effects of ecdysterone *in vitro*. *Science*, **167**: 61–62.

MARKS, E. P., AND R. A. LEOPOLD, 1971. Deposition of cuticular substances *in vitro* by leg regenerates from the cockroach, *Leucophaea maderae* (F.). *Biol. Bull.*, **140**: 73–83.

MARKS, E. P., AND J. P. REINECKE, 1965. Regenerating tissue from the cockroach, Leucophaea maderae: Effects of endocrine glands *in vitro*. *Gen. Comp. Endocrinol.*, **5**: 241–247.

OBERLANDER, H., 1969. Effects of ecdysone, ecdysterone, and inokosterone on the *in vitro* initiation of metamorphosis of wing discs of *Galleria mellonella*. *J. Insect Physiol.*, **15**: 297–304.

OBERLANDER, H., AND L. FULCO, 1967. Growth and partial metamorphosis of imaginal discs of the greater wax moth, *Galleria mellonella in vitro*. *Nature*, **216**: 1140–1141.

OBERLANDER, H., AND C. TOMBLIN, 1972. Cuticle deposition in imaginal discs: Effects of juvenile hormone and fat body *in vitro*. *Science*, **177**: 441–442.

O'FARRELL, A., AND A. STOCK, 1953. Regeneration and the molting cycle in *Blattella germanica* (L.). I. Single regeneration initiated during the first instar. *Aust. J. Biol. Sci.*, **6**: 485–500.

OHTAKI, T., R. MILKMAN AND C. WILLIAMS, 1968. Dynamics of ecdysone secretion and action in the flesh fly *Sarcophaga peregrina*. *Biol. Bull.*, **135**: 322–334.

ROSE, G., 1954. A separable and multipurpose tissue culture chamber. *Tex. Rep. Biol. Med.*, **21**: 1074–1083.

SCHNEIDER, I., 1966. Histology of larval eye-antennal discs and cephalic ganglia of *Drosophila* cultured in vitro. *J. Embryol. Exp. Morphol.*, **15**: 271–279.

SOCHA, R., AND F. SENHAL, 1972. Inhibition of adult development in *Tenebrio molitor* by insect hormones and antibiotics. *J. Insect Physiol.*, **18**: 317–337.

WILLIAMS, C. M., 1968. Ecdysone and ecdysone-analogues: Their assay and action on diapausing pupae of the *Cynthia* silkworm. *Biol. Bull.*, **134**: 344–355.

Reference: *Biol. Bull.,* **145**: 180–199. (August, 1973)

SALT AND WATER BALANCE IN LUGWORMS (POLYCHAETA: ARENICOLIDAE), WITH PARTICULAR REFERENCE TO *ABARENICOLA PACIFICA* IN COOS BAY, OREGON

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Polychaetes of the family Arenicolidae, commonly called lugworms, are important members of the infauna of muddy-sand beaches throughout the world, both in estuaries and along the open coast where wave action is slight (Wells, 1963). On suitable beaches, lugworms may be so abundant that their burrowing and feeding activities markedly influence the composition and structure of the substrate (Clay, 1959; Klein, 1967), much as do certain other mudflat polychaetes studied quantitatively by Rhoads (1967). Lugworms are of great importance in processing organic detritus and debris in estuaries (Clay, 1959; Day, 1967; Longbottom, 1970), and provide food for birds (Clay, 1959; Orton, 1925), and bottom-feeding fishes of both sport and commercial importance (Clay, 1959; Day, 1967). Lugworms are therefore a significant link in the detritus food web that is characteristic of estuaries (Darnell, 1967).

Lugworms have been subjects of numerous studies on morphology, feeding and burrowing activities, respiratory physiology, reproduction, and other aspects of their ecology and physiology. Most of these studies have dealt with the common European lugworm, *Arenicola marina* (Linnaeus), while other species of the family have received little attention. These studies have been reviewed several times, initially by Ashworth (1904), and more recently by Wells (1945), Clay (1959), Green (1968) and Krüger (1971). In view of their extensive distribution in estuaries, it is surprising that salt and water balance in lugworms has been so little studied. While there are many separate reports on various aspects of this topic (see Discussion), most of these provide ony fragmentary data which do not give a comprehensive picture of any species.

Several species of *Abarenicola* are the common lugworms of estuaries and quiet coastal waters around the North Pacific Ocean from Japan to northern California (Healy and Wells, 1959). *Ab. pacifica* Healy and Wells is one of the most common polychaetes in Coos Bay, the largest estuary in Oregon. Because of the abundance and presumed ecological importance of *Ab. pacifica,* and because of the limited published information on salt and water balance in lugworms generally, the present study was conducted.

MATERIALS AND METHODS

The nomenclature for the Arenicolidae follows Wells (1959, 1963). The abbreviations *A.* will be used for the genus *Arenicola, Ab.* for *Abarenicola,* and *Ad.* for *Arenicolides.*

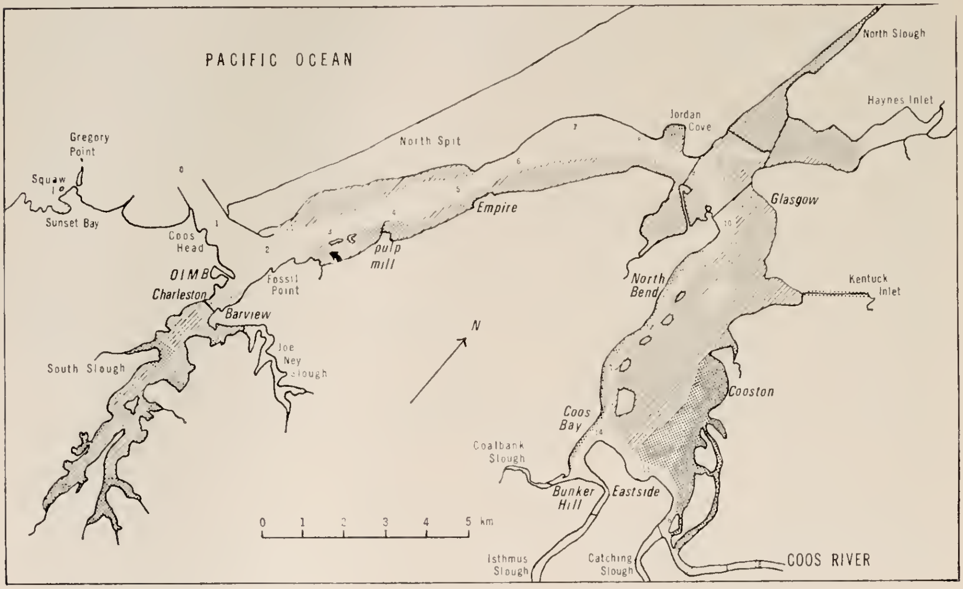
Coos Bay (Fig. 1) is a drowned river mouth of relatively recent geological origin, with a mean high tide area of 10,500 acres, reduced to about 5000 acres at mean low tide. Estuarine effects are detectable about 30 miles upstream in the Coos River system, the only significant source of fresh water to the estuary. In summer and fall, with low river flow, salinities in Coos Bay are high. Salinities lower than 25‰ were not encountered below mile marker 14 in October 1957 (Burt and McAlister, 1959), and below mile marker 10 in summer 1930–32 (U. S. Department of Interior, 1971). In summer 1970, interstitial salinities as high as 28‰ were measured at mile marker 15, in the Coos River. At this time of year the entire Coos Bay estuary is essentially of the well-mixed type (Burt and McAlister, 1959), and complete vertical mixing is further encouraged by tidal overmixing, a phenomenon which was originally described from this Bay (Burt and Queen, 1957). Only in the channel of Coos River itself is there any vertical salinity stratification (Burt and McAlister, 1959). There are no published records of salinity patterns in Coos Bay during winter and spring when river flow is much greater than in summer. The presence of many stenohaline, sessile marine organisms inside the mouth of Coos Bay, especially on the rocks of Coos Head and near Fossil Point (Fig. 1) suggests that at least the lower portions of the Bay remain dominated by marine water throughout the year.

From the original descriptions by Healy and Wells (1959), and from the ecological and behavioral study by Hobson (1967), one would expect two species of lugworms to be present in Coos Bay: *Ab. pacifica* and *Ab. claparedi* (Levinsen) subsp. *vagabunda* Healy and Wells. However, there are no confirmed reports of *Ab. claparedi vagabunda*. While *Ab. pacifica* was found to be widely distributed and frequently abundant, *Ab. claparedi vagabunda* was not reported in an extensive survey of Coos Bay made by students of the Oregon Institute of Marine Biology during the summer of 1970 (Porch, 1970; U. S. Department of the Interior, 1971). In summer 1970, *Ab. pacifica* was present in nearly all parts of Coos Bay from near the estuary mouth to near the mouth of Coos River at mile marker 15, as well as in a small patch of muddy sand on the marine beach at Sunset Bay (Fig. 1).

For physiological study, lugworms were collected from an extensive intertidal mudflat near Pigeon Point (Fig. 1). Studies by students of the Institute of Marine Biology in the summer of 1969 indicated that circulation patterns in this area were such that the population of worms sampled was seldom exposed to effluent from the pulp mill at mile marker 4. Interstitial salinities were always high, about 95–96% of International Normal Sea Water (SW) of 560 mM Cl⁻ (Oglesby, 1969a). Worm burrows were similar to those described for *A. marina* (Wells, 1945) and for *Ab. pacifica* and *Ab. claparedi vagabunda* by Hobson (1967). Densities ranged up to more than 100 worms per square meter. Worms of all sizes were present in the population, ranging from 1–2 cm long up to 8–10 cm in length, weighing over 4 g. The length of the tail portion, relative to the rest of the body, varied considerably, suggesting extensive partial predation by fish or birds, as described by Orton (1925) for *A. marina*.

After collection, lugworms were maintained in non-circulating seawater at 14–16°, without feeding. Glass tubes of appropriate size were provided as artificial burrows, and most worms took up residence in the tubes, performing regular periodic irrigation movements. Lugworms were adapted to various salinities by stepwise dilution. Experimental media were made with fresh seawater from the laboratory system (salinity 95–96% SW) diluted with glass-distilled water. The medium was changed daily to prevent fouling; aeration was not provided. Lugworms were adapted to the test salinity for at least a week before study. The lugworms in 40% and 29% SW were sampled after only 4 days in the final adaptational medium, since they were in poor condition and it seemed unlikely that they would have survived a full week in such low salinites.

Ten lugworms were sampled at each salinity. Coelomic fluid was taken from the worms as described by Oglesby (1968b) for sipunculids, care being taken to avoid contamination with gut fluids or blood, and centrifuged to eliminate all cellular material. Measurements of Cl⁻ were done with a Cotlove chloridometer (Oglesby, 1968b). Measurements of Na⁺ were done with an EEL flame photometer (Oglesby, 1970). Osmotic concentrations were measured with a Mechrolab vapor pressure osmometer (Oglesby, 1968b), and expressed as the millimolarity of an equivalent NaCl solution. For water analysis, worms were dried to constant weight.


PACIFIC OCEAN
North Slough
Haynes Inlet
Jordan
Cove
North Spit
Gregory
Point
Squaw
Is
Sunset Bay
Coos
Head
OIMB
Charleston
Barview
Fossil
Point
pulp
mill
Empire
Glasgow
North
Bend
Kentuck
Inlet
N
South Slough
Joe
Ney
Slough
Cooston
Coos
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Bunker
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Eastside
0 1 2 3 4 5 km
Isthmus
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0
1
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14

FIGURE 1. Map of Coos Bay, Oregon, showing collecting site for *Abarenicola pacifica* (black arrow). Intertidal areas where lugworms were recorded in 1969–70 are indicated by diagonal lines; intertidal areas of apparently suitable substrate where no lugworms were reported in 1969–70 are indicated by stippling; intertidal areas of unsuitable substrate (coarse sand, rocks, *etc.*); subtidal areas, and areas not investigated are left blank. Numbers indicate navigation mile markers for main ship channel. Names of towns are italicized.

Results are expressed as the mean ±1 standard deviation, indicated on the figures by vertical bars. Unless otherwise stated, statistical significance is considered at the 95% level of confidence, using Student's *t*-test.

RESULTS

The osmotic concentrations of centrifuged coelomic fluids of *Ab. pacifica* adapted to salinities from 29% SW to 97% SW are presented in Figure 2, Cl^- concentrations in Figure 2, and Na^+ concentrations in Figure 3. Coelomic fluid/medium ratios for osmotic, Cl^-, and Na^+ concentrations are presented in Figure 4.

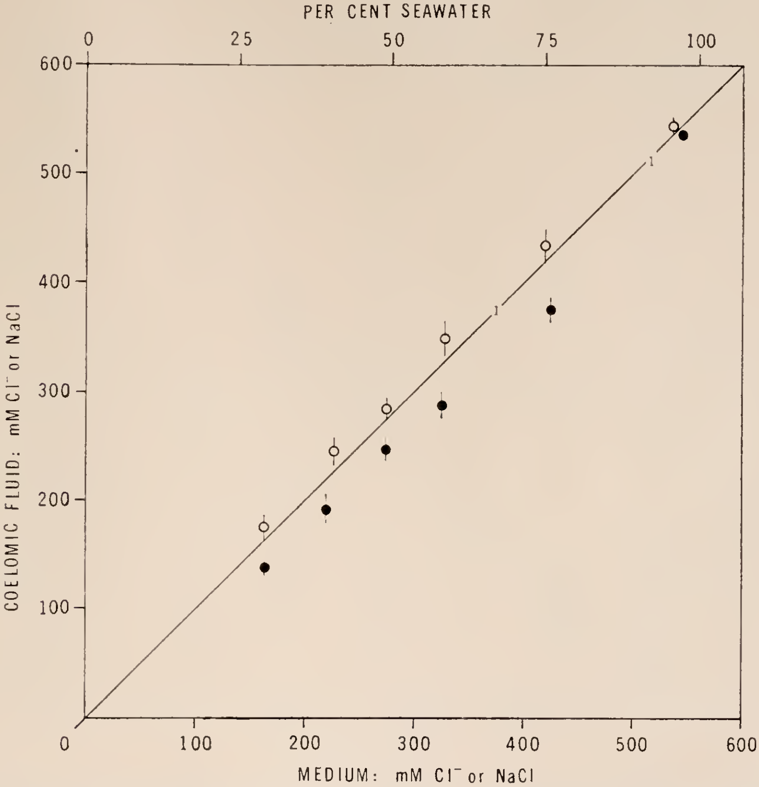

FIGURE 2. Relationship of osmotic and chloride concentration in coelomic fluid of *Ab. pacifica* to Cl^- concentration of external medium. Diagonal line indicates equal internal and external concentration; solid circles, Cl^- concentrations; open circles, osmotic concentrations; Cl^- concentrations for *Arenicola marina:* 1, from Robertson (1949).

The coelomic fluid of *Ab. pacifica* is slightly, but significantly, hyperosmotic to the medium at all salinities tested, by 5.1% ($CF/M = 1.05 \pm 0.049$; $N = 60$). There is no indication of increasingly hyperosmotic regulation by lugworms adapted to the lower salinities. The coelomic fluid is slightly, but significantly, hypo-ionic to the medium at all salinities with respect both to Cl^-, by 10.2% ($CF/M = 0.898 \pm 0.057$; $N = 60$), and to Na^+, by 2.9% ($CF/M = 0.971 \pm 0.030$; $N = 60$). There are no statistically significant trends in changes of either osmotic or Na^+ concentrations with lowering of external salinity. However, the Cl^- concentration of the coelomic fluid of lugworms adapted to 97% SW is significantly higher (CF/M = 0.988 ± 0.010; N = 10) than for the worms adapted to lower salinities (CF/M = 0.880 ± 0.044; N = 50). Even in the lugworms adapted to 97% SW, the internal Cl^- was significantly hypo-ionic to that of the medium by 1.2%. There was no significant difference in the extent of this slight hypo-ionic condition between lugworms adapted to 97% SW for 5 days in the laboratory and those adapted for 13 days.

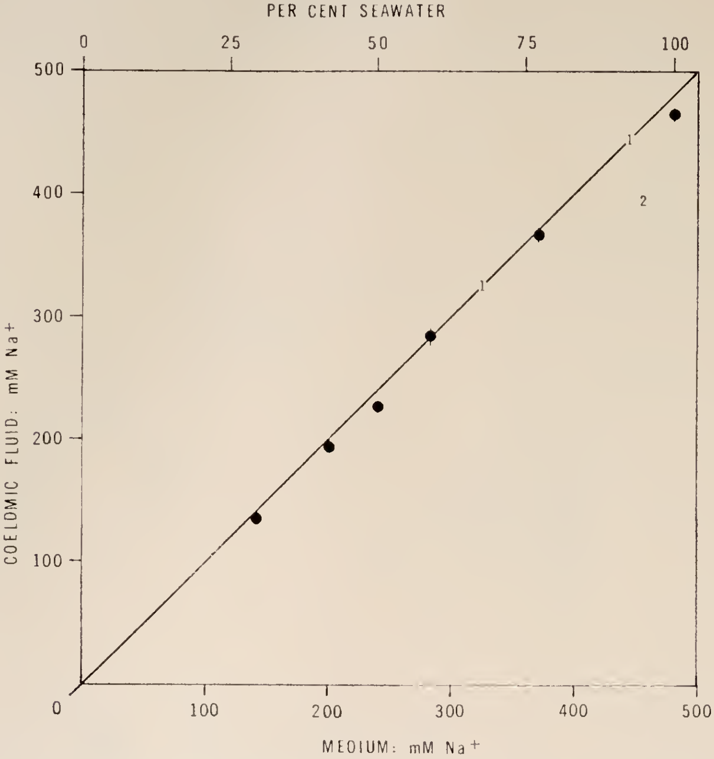

FIGURE 3. Relationship of Na^+ concentration in coelomic fluid of *Ab. pacifica* to Na^+ concentration of external medium. Diagonal line indicates equal internal and external concentration; Na^+ concentrations for *Arenicola marina:* 1, from Robertson (1949); 2, from Nesterov and Skulski (1965).

Figure 5 presents the results of water content determinations of *Ab. pacifica* adapted to a wide range of salinities. A single regression line (method of least squares) was fitted to the data. The results show that the water content of *Ab. pacifica* is strongly dependent upon the external salinity: the lower salinity, the more hydrated the worms become.

DISCUSSION

Abarenicola pacifica is an osmotic conformer over the entire salinity range tested (29% to 96% SW). Over this entire salinity range, the coelomic fluids are slightly, but significantly, hyperosmotic to the external medium by about 5%. At 100% SW, this would correspond to an osmotic pressure difference of about 1.2 atm., and at 50% SW, to an osmotic pressure difference of about 0.65 atm. While these are the first measurements of osmotic concentration in *Ab. pacifica*, there are several previously published data on two other species, *A. marina* and *Ad. branchialis* (Audouin and Milne Edwards); the available data on osmotic concentrations for all three species are graphed in Figure 6. It should be pointed out that some of these data were obtained for worms which had not been adapted for more than a few hours to experimental salinities. However, in the one study of the time course of changes in coelomic fluid concentrations after a transfer to a lower salinity, Beadle (in Wells and Ledingham, 1940) found that effective iso-osmoticity was reached in no more than 7 hours by *A. marina*.

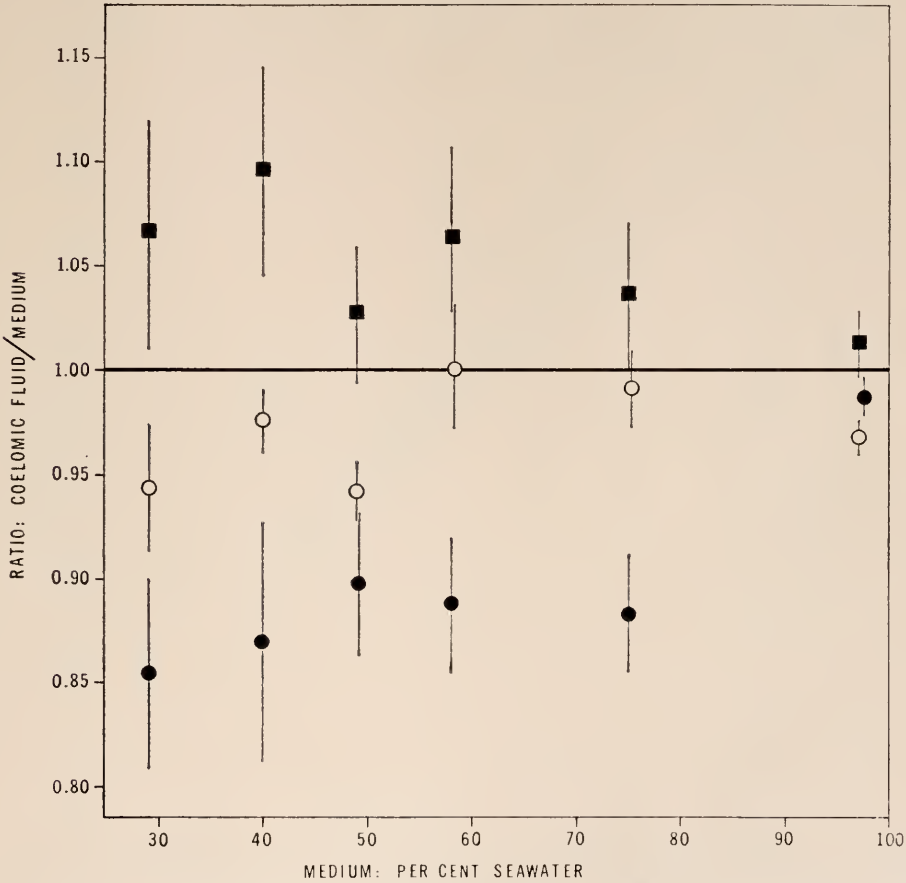

FIGURE 4. Relationships of coelomic fluid/medium (CF/M) ratios for osmotic concentration (solid squares), Na^+ concentration (open circles), and Cl^- concentrations (solid circles) in *Ab. pacifica* to concentration of external medium, expressed as percentage sea water. Horizontal line indicates equal internal and external concentration.

The collective data for *A. marina* give the impression that this species is similar to *Ab. pacifica* in being an osmoconformer with the coelomic fluids slightly hyperosmotic to the external medium. The data given by Beadle (in Wells and Ledingham, 1940), Duchâteau-Bosson, Jeuniaux and Florkin (1961), Ginetzinsky (1959), Quinton (1900), and Schlieper (1929) are all within a few per cent of being iso-osmotic. Primarily on the basis of Schlieper's data, the conclusion that *A. marina* is an iso-osmotic osmoconformer has been repeated in later reviews (*e.g.* Green, 1968; Krogh, 1939; Oglesby, 1969a; Potts and Parry, 1964). However, Belyaev's (1957) results show a stronger degree of hyperosmoticity, by an average of 9% for the Barents Sea population and an average of 12% for the White Sea population, with some data points ranging as high as 24% more concentrated than the medium. There is some difficulty in interpreting Belyaev's

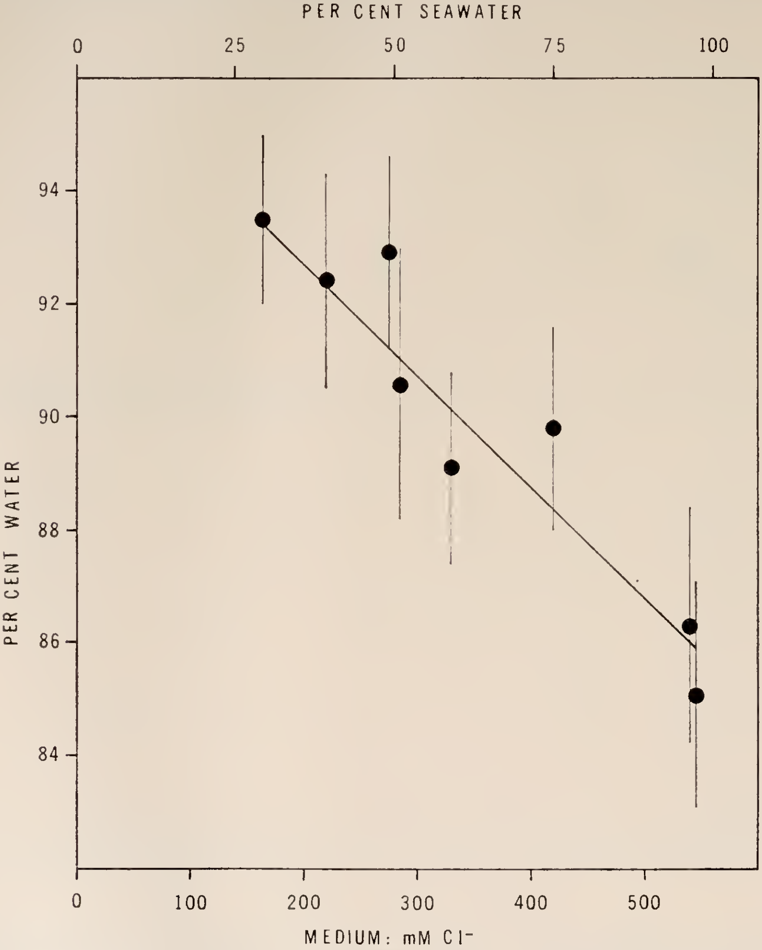

FIGURE 5. Relationship of water content of *Ab. pacifica* to Cl^- concentration of medium.

Figure 9, from which these data were taken, because the freezing point depressions for the media do not correspond to their stated salinity [the relationship, according to Krogh (1939), should be −1.0° C = 293 mM NaCl = 18.3‰ S]. Belyaev's freezing point depression values were taken and regraphed for use in Figure 6.

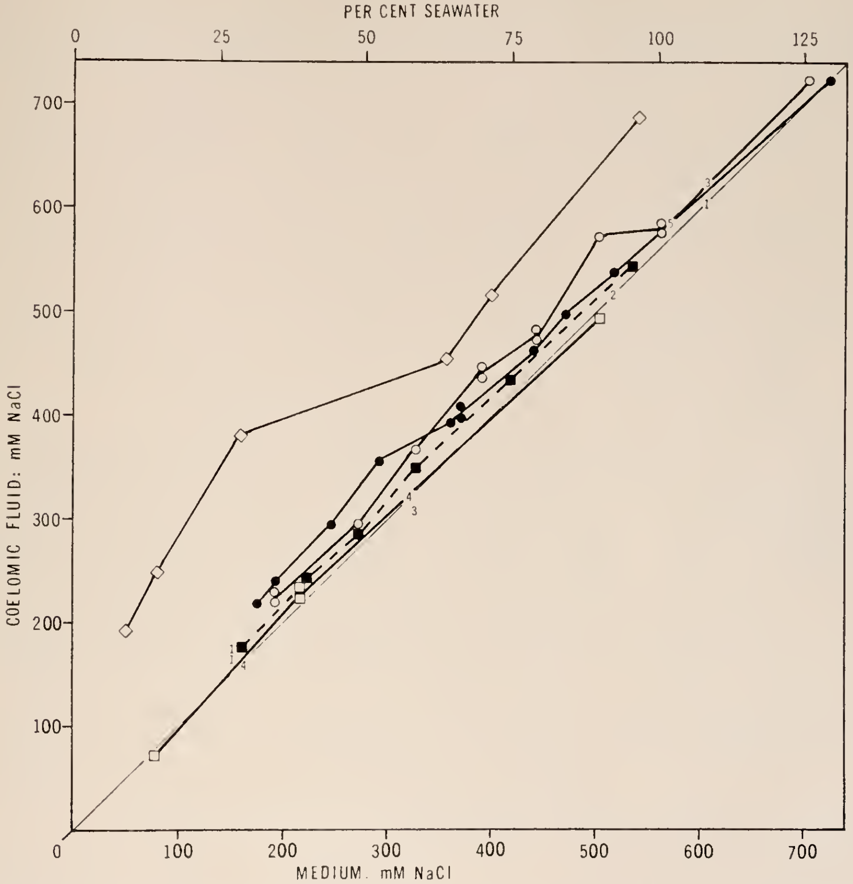

FIGURE 6. Relationship of osmotic concentrations in coelomic fluids of lugworms to osmotic concentrations of external medium: *Arenicola marina:* solid circles, White Sea (Belyaev, 1957); open circles, Barents Sea (Belyaev, 1957); open squares, Kiel and Helgoland (Schlieper, 1929); 1, Plymouth and Bangor (Beadle, in Wells and Ledingham, 1940); 2, Naples (Quinton, 1900); 3, Roscoff (Duchâteau-Bosson, Jeuniaux, and Florkin, 1961); 4, White Sea (Ginetzinsky, 1959); 5, (Krukenberg, in Ashworth, 1904); *Arenicolides branchialis:* open diamonds, Sea of Azov (Zenkevich, 1938a, 1938b); *Abarenicola pacifica:* solid squares, Coos Bay (this report).

Belyaev's interpretation of his results is that both populations of *A. marina* are hyperosmotic regulators at or near their habitat salinities, which were different for the two populations. However, his results as regraphed in Figure 6 do not show any distinct "plateau" of hyperosmotic regulation such as that which characterizes nereid polychaetes (Oglesby, 1969a). Belyaev's results actually seem more consistent with the conclusion that *A. marina* is a hyperosmotic osmoconformer at all salinities.

Zenkevich's (1938a, 1938b) results for *Ad. branchialis* (as *A. grubei*) present even greater problems for interpretation. As regraphed in Figure 6 (using Zenkevich's data for worms after 25 hours acclimation), *Ad. branchialis* appears to be strikingly hyperosmotic to the medium at all salinities, and shows a marked "plateau" region suggestive of active hyperosmotic regulation between about 25% and 50% SW. Not only are these results different from those for *A. marina* and *Ab. pacifica,* they are different from those for any other polychaete (Oglesby, 1969a). As with Belyaev's Figure 9, the stated enviromental salinities in Zenkevich's (1938b) Figure 15 do not correspond to the measured freezing point depressions. Zenkevich (1939b) found marked temporal variations in freezing point depressions after 7, 12 and 25 hours in the experimental salinities, but these variations were not proportional to either the length of the acclimation time or to the salinity. As discussed by Oglesby (1969a), all measurements of freezing point depressions made by Zenkevich (1938a, 1938b) in a wide variety of organisms were markedly hyperosmotic to the "average" environmental salinity in the Sea of Azov. Conflicting data by other workers who have investigated these same or similar species cast doubt on the validity of Zenkevich's results.

It can be concluded, therefore, that both *Ab. pacifica* and *A. marina,* and probably all members of the family Arenicolidae, are osmotic conformers which maintain a slight degree of hyperosmoticity over a fairly wide salinity range. The osmotic behavior of lugworms is thus quite unlike that of many estuarine invertebrates, such as most nereid polychaetes (Oglesby, 1969a), the amphipod *Gammarus duebeni* (Sutcliffe, 1967), and several decapod crustaceans (Gross, 1964), all of which are osmotic conformers only at higher salinities, and are active hyperosmotic regulators at salinities lower than about 25% SW. Rather, lugworms are osmotically similar to the ribbed mussel *Modiolus demissus* (Pierce, 1970), the bay mussel *Mytilus edulis* (Gilles, 1972), and the acorn barnacle *Balanus improvisus* (Newman, 1967), as well as the polychaetes *Nereis vexillosa* (Oglesby, 1965) and *Cirriformia spirabrancha* (Dice, 1969). All are osmotic conformers and all can tolerate a wide range of external salinities; this tolerance permits at least some to penetrate far into the oligohaline reaches of estuaries.

It has only recently been realized that many marine and estuarine animals, usually considered to be iso-osmotic, in fact maintain a small but statistically significant degree of hyperosmoticity of the body fluids to the environment (Dice, 1969; Pierce, 1970; Remmert, 1969), as was remarked upon as long ago as 1935 by Nicol. Pierce (page 530) believes that "any animal having protein in solution in its extracellular water and permeable external membranes must be hyperosmotic to its environment," this condition being the purely passive consequence of the presence of osmotically active but indiffusible particles such as proteins in the body fluids resulting in a Gibbs-Donnan equilibrium. Pierce (1970) suggested that the usually small differences in osmotic concentrations between the body fluids and the external media had hitherto gone unrecognized because earlier workers had used less precise methods than are now available. Pierce's conclusion, while theoretically attractive, may not be true for all real animals, even when one excludes from consideration such hypo-osmotic regulators as marine teleost fishes, many crabs, and the brine shrimp *Artemia*. Most sipunculid worms, for example, seem clearly to be isosmotic conformers at all tolerable salinities (Oglesby, 1969a; Hogue and Oglesby, 1972). The one reported measurement of plasma protein concentrations in lugworms is only 0.2 g/1 (in *A. marina:* Robertson, 1949), a value at the low end of the range of plasma protein measurements in body fluids of those worms lacking a dissolved respiratory pigment (Oglesby, 1969a). Robertson (1949) carried out dialysis experiments of body fluids against sea water using collodion membranes, and concluded that a Gibbs-Donnan equilibrium was not significant in many marine invertebrates, including *A. marina*.

Remmert (1969, page 424) offered no explanation for his conclusion that "osmotic conformers are slightly hyperosmotic in their normal environment." Pierce (1970) discussed aspects of the differences in osmotic pressures between blood and pericardial fluids in bivalve molluscs, but did not address himself to the ecological role, if any, of the blood's being hyperosmotic to the external environment. Dice (1969) suggested that the maintenance of hyperosmotic coelomic fluids in marine annelids caused a constant osmotic influx of water into the worm which could replace water lost in the urine. Strunk (1930a) and Chapman and Newell (1947) have described fairly copious urination by *A. marina,* so the necessity for replacing urinary water is present. Dice's (1969) suggestion seems quite likely as at least a partial explanation for the adaptive value of slightly hyperosmotic body fluids in marine and estuarine osmoconformers.

Na^+ and Cl^- concentrations are slightly lower in the coelomic fluids of *Ab. pacifica* than in the external medium at all salinities, by about 3% and 10%, respectively (Figs. 2, 3). In the case of Cl^-, the CF/M ratio was significantly lower in worms adapted to salinities below the habitat salinity of 97% SW. Robertson (1949) and Nesterov and Skulski (1965) have measured Na^+ in the coelomic fluids of *A. marina,* and Robertson has also reported values for Cl^-; these are indicated by small numbers in Figures 2 and 3. Robertson's data indicate that this species has coelomic fluids that are virtually iso-ionic with respect to both Cl^- and Na^+, and that this situation does not change after a short exposure to lowered salinity. On the other hand, Nesterov and Skulski's measurement of Na^+ is 14% lower in the coelomic fluid than in the medium.

Bialaszewicz (1933) gave data for several ions, including Cl^- but not Na^+, in the coelomic fluids of *Ab. claparedi.* Since he did not report the ionic composition of the external medium (about 112% SW), it is not possible to compute exact CF/M ratios for any of the ions he measured. The ratios discussed below were calculated on the assumption that the coelomic fluids of *Ab. claparedi* are iso-ionic with respect to Cl^- (see Oglesby, 1969a).

Bialaszewicz (1933), Nesterov and Skulski (1965), and Robertson (1949) have reported on several additional ions in *A. marina* and *Ab. claparedi.* Among the cations, K^+ was reported by all three workers to be hyper-ionic in both species, by 2.5–39.7%. Robertson observed that the CF/M ratio for K^+ increased from 1.035 to 1.18–1.20 after *A. marina* had been briefly exposed to about 50% and 67% SW. (But see Oglesby, 1970, and Hogue and Oglesby, 1972, for critical discussion of the significance and reliability of apparently elevated K^+ concentrations in worm body fluids.) The reported value of 0.0214 mM Li^+ for *A. marina* (Nesterov and Skulski) suggests that this ion is present in the coelomic fluid at about 1.5 times the concentration in normal seawater. Ca^{++} is reported to be 0.2% lower in the coelomic fluid of *A. marina* than in the medium (Robertson), but 17.2% higher in *Ab. claparedi* (Bialaszewicz). Again, Robertson observed that the CF/M ratio for Ca^{++} rose from 0.998 to 1.12–1.13 after the worms were exposed to 50% and 67% SW. Mg^{++} was reported to be 0.3% higher in the coelomic fluid of *A. marina* than in the medium (Robertson), but 0.9% lower in *Ab. claparedi* (Bialaszewicz). Both Bialaszewicz and Robertson found somewhat reduced $SO_4^=$ concentrations (8.2% and 7.8%, respectively). Robertson's dialysis experiments showed that CF/M differences for K^+ and $SO_4^=$ were not the result of Gibbs-Donnan effects caused by the presence of proteins. It cannot be stated whether the differences between Bialaszewicz's and Robertson's measurements for most of these ions represent valid specific differences, or whether they are the result of different analytical methods. In none of these studies was the statistical significance of the slight deviations from iso-ionic conditions evaluated.

Taken together, these data do not indicate any marked ionic regulation in lugworms. However, some of these aniso-ionic situations, though slight, may be real; for example, Cl^- and Na^+ in *Ab. pacifica,* and K^+ and $SO_4^=$ in *Ab. claparedi* and *A. marina.* With the exception of K^+, the concentrations of these ions in the coelomic fluids are lower than in the external medium, even though the coelomic fluids are hyperosmotic to the medium. This suggests the presence of an ion deficit in the coelomic fluids of lugworms, particularly marked for anions, which is perhaps made up with organic, or nitrogenous substances, or both.

There have been very few measurements of organic substances in the body fluids of any worms (Oglesby, 1969a), and almost none among lugworms. It is reported that *A. marina* has from 0.25–1.83 mM urea (Strunk, 1932), only 0.00059 mM uric acid (Strunk, 1930b), and 0.67 mM glucose and other reducing sugars (Florkin, 1936). Allantoic acid was detected, but not quantified, in coelomic fluid of *A. marina* (Florkin and Houet, 1939). More extensive data are given by Clark (1968a, 1968b) for *Ab. pacifica.* She reported 0.48 ± 0.15 mM NH_3, and gave 12 pooled measurements for free amino acids (FAA) for worms in three different salinities, for different periods of adaptation to these salinities, and for different portions of the body. For all groups of worms in all salinities, the average value was 10.18 ± 4.98 mM FAA. There were no significant differences between the worms in 100%, 75%, and 50% SW ("100% SW" = 30–32‰ Salinity), for either 1 or 4 days adaptation. The highest single value, 19.78 mM FAA, was the one measurement of coelomic fluid from the tail region, all other measurements being from the trunk. These FAA values are much lower than those Clark reported from many other polychaetes in the same study, which ranged up to 100 mM FAA in the terebellid *Thelepus crispus.* Clark (1968a) also observed differences in pH between the coelomic fluids of tail and trunk regions of *Ab. pacifica.* Using pooled samples from 7 worms, she found the tail coelomic fluid had a pH of 7.49, but that the coelomic fluid from the trunk had a lower pH, 7.30. These values compare well with the only other reported pH measurement for a lugworm, 7.43 ± 0.059 in *Arenicola cristata* Stimpson (Mangum and Shick, 1972).

Adding up all these separate values for both species, 22.76 mM can be accounted for by organic and nitrogenous molecules if Clark's maximum value for FAA in

Ab. pacifica is used, and only 13.16 mM if Clark's average value is used. Such low concentrations could contribute only 1–2% to the total osmolarity in *Ab. pacifica* adapted to 100% SW, and thus are not sufficient to make up the entire difference between the osmotic and ionic concentrations in the coelomic fluid of this species. Some additional osmotically active substance(s) must therefore be present in the coelomic fluids of lugworms.

Ab. pacifica increases markedly in water content when adapted to salinities lower than 97% SW (Fig. 5). For example, a lugworm with 14.4 g solid material (dry weight) would have 85.6 mg water when adapted to 100% SW, but would have 135.8 mg water when adapted to 50% SW, representing an increase of 159% over the water content of the lugworms in the higher salinity. If this lugworm could be adapted to as low a salinity as 25% SW, the 14.4 mg solid material would be associated with 228.6 mg water, a 267% increase in water content over the worms adapted to 100% SW. This dependency of water content upon external salinity is at least as strong as in *Nereis vexillosa* and *Themiste dyscritum,* both species with a far less extensive distribution in estuaries (Oglesby, 1965, 1968b, 1969a). In an analysis of water-content regulation in various worms (Oglesby, 1973), it is shown from the present data that *Ab. pacifica* retains about 65% of the excess water which would be taken up osmotically by a theoretical "perfect osmometer" after transfers to lower salinities; even *T. dyscritum* is capable of preventing the retention of more of this excess osomotic water than *Ab. pacifica.* These observations on steady-state water content regulation are supported by visual observations of extensive swelling associated with transfers of lugworms to lower salinities, accompanied by distention and even breakage of the body wall. Swollen worms were incapable of making normal ventilatory movements, and would be expected to have difficulty in burrowing. Below 50% SW, these abnormal responses were especially noticeable.

Very few investigators have addressed themselves to the problem of water regulation in lugworms, and what information exists was obtained quite incidentally to other studies. There have been several reports of water content in *A. marina* and *A. cristata* (see Table I, Oglesby, 1969a), and although all worms were probably taken from approximately full-strength seawater, in no case was the external salinity indicated. The reported values have an extraordinary range, from 78.6% to 97.0% water just for one species (*A. marina*). Both extremes are well outside the range of water content in all salinities reported here for *Ab. pacifica.* Probably these data should be used with caution until more carefully controlled work is done with *A. marina.*

The tendency for a worm to approach its initial weight after a transfer from one salinity to another is termed "volume regulation" (Oglesby, 1969a). No studies have been published on the actual time course of weight changes after transfers, but scattered comments in the literature indicate that *A. marina* (Dakin, 1908; Ginetzinsky, 1959; Quinton, 1900; Reid, 1929), *Ad. branchialis* (Zenkevich, 1938a); and *Ab. pacifica* (Clark, 1969b; this report) have little or no capacity for volume regulation, suggestions which are consistent with the great variation in steady-state water content observed in *Ab pacifica.* It can reasonably be concluded that lugworms have a very limited capacity to regulate water content when exposed to variations in environmental salinity.

The studies just discussed indicate that both *Ab. pacifica* and *A. marina,* and very likely all lugworms, are hyperosomotic osmoconformers which cannot regulate volume and water content to any significant extent. When maintained in salinities lower than about 50% SW in the laboratory, lugworms are rendered incapable of such vital physiological activities as burrowing and burrow ventilation. Yet *Ab. pacifica* has a very extensive distribution in the Coos Bay estuary, which has some freshwater inflow at all seasons, and very strong freshwater inflows from the Coos River during the rainy season (November–May). Similarly, *A. marina* is generally regarded as a typical inhabitant of estuaries in northern Europe (*e. g.,* Green, 1968). In view of their relatively poor physiological abilities to control salt and water balance in the laboratory, how do lugworms cope with the rigorous conditions of varying salinities in estuaries?

TABLE I

Tolerance of low salinities by lugworms

External concentration mM Cl⁻	Remarks and references
Arenicola marina	
50	Survival only "for a short time" (Reid 1929).
80–85	Serious injuries occur below this level (Khlebovich 1969); survived 26 hours in laboratory (Schlieper 1929).
125	Lowest temporary salinity at which worms were found in field (Nicol 1935); lowest salinity for worms in field (Green 1968; Krogh 1939; Wells and Ledingham 1940).
140–193	Survived only 2 hours in laboratory (Pearse 1928); survived 7 hours in laboratory (Beadle in Wells and Ledingham 1940); no tolerance of lower salinities in laboratory (Belyaev 1957; Ginetzinsky 1959; Sveshnikov 1963).
230	Lowest salinity permitting normal burrowing (Reid 1929).
344–392	Lowest salinity for worms in field (Percival 1929; Popham 1966; Robertson 1949).
Arenicolides branchialis	
48	Survived 25 hours in laboratory (Zenkevich 1938b).
357	Average environmental salinity (Zenkevich 1938b).
Abarenicola pacifica	
162–220	Poor survival in laboratory (this report).
247–277	Survived 4 days in laboratory (Clark 1968b); moderate survival in laboratory (this report).
400	Lowest interstital salinity at which worms were found in field (this report).

The actual tolerance of lugworms to low salinities may not be so great as generally believed. Table I gives a number of records of low salinity tolerances for 3 species of lugworms; undoubtedly other low salinity limits have been published, especially in faunal lists, but I hope that this selection is representative. Table I gives a first impression that lugworms can tolerate salinities down to at least 23% SW (8‰S) in the field, and much lower salinities in the laboratory. However, in the laboratory lugworms show obvious signs of injury when exposed to salinities below about 25% SW for more than a few hours. Schlieper's (1929) record for *A. marina* surviving 26 hours in 15% SW and Zenkevich's

(1938b) record for *Ad. branchialis* surviving 25 hours in 9% SW are by far the longest recorded laboratory survivals of lugworms in salinities below 23% SW. *Ab. pacifica* placed in salinities lower than about 50% SW recovered if returned to higher salinities within 1–2 days. Thus, in this species, brief exposures to low salinities do not cause irreversible damage.

A salinity of 5–8‰ is frequently regarded as representing an important boundary for the low salinity tolerance of euryhaline estuarine osmoconformers (review; Khlebovich, 1969). This critical low salinity limit was termed the "horohalinicum" by Kinne (1971). The horohalinicum is not a tolerance boundary for such estuarine worms as nereid polychaetes, but it seems significant that 5–8‰S is approximately the external concentration at which these worms abandon osmoconformity and begin to hyperosmoregulate (Oglesby, 1969a). The horohalinicum of natural waters seems to be characterized by marked changes in ion ratios, which are constant throughout the entire range of higher salinities. At and below the horohalinicum, K^+ and Ca^{++} tend to become much more abundant relative to Na^+ than in higher salinities (Khlebovich, 1969; Kinne, 1971). Thus, the several observations that lugworms can survive up to 2 days in salinities below 8‰ in the laboratory may be a fortunate consequence of the use of distilled water to make seawater dilutions, leaving the various ion ratios unaltered. However, for the osmoregulator *Nereis diversicolor*, Ca^{++} must be present in the external medium for survival in salinities below the horohalinicum (Oglesby, 1970; Smith, 1970).

While there are a number of references to lugworms being found exposed to salinities as low as 8‰ in the field (Table I), suggesting that lugworms can exist naturally down to the horohalinicum, many low salinity records seem to be of the type described by Nicol (1935). She states (page 220) that *A. marina* is "found in [salt marsh] pools of an average salinity as low as 15‰. In the pools the salinity does not often fall below 8‰." Nicol's observations may well be the actual basis for later undocumented and unqualified statements, such as that *A. marina* "can tolerate reduced salinities down to about 8‰" (Green, 1968) (*e.g.*, also Krogh, 1939; Wells and Ledingham, 1940). Nicol clearly stated that these reductions of salinity were brief and temporary, due to rainfall or to surface runoff, and that they did not influence interstitial water salinity to any extent even when the salinity of the overlying water was considerably reduced.

That burrowing animals may be able to avoid or reduce contact with overlying waters of low salinity for short periods of time, while remaining in contact with interstitial water of much higher salinity, is discussed in some detail by Oglesby (1969b). Such burrowers will be limited by how long they can tolerate the absence of new water for burrow ventilation. In the case of lugworms, such periods may be quite long. May (1972, page 80) observed that *Ab. pacifica* "appeared healthy after three days in anoxia," and Hecht (1932) maintained *A. marina* for 9 days in the absence of oxygen. Thus, lugworms are capable of "riding out" short periods of lowered environmental salinities in much the same way as can barnacles (Newman, 1967) and bivalve molluscs (Gilles, 1972), which prevent contact of the body with external medium by closing their shells. MacGinitie (1939), for example, reported that a number of soft-bodied burrowing invertebrates, including *A. brasiliensis* Nonato (as *A. cristata*), survived a major freshwater flood in the normally marine Newport Bay in southern California, while epifaunal forms lacking shells were killed. Just how effectively a lugworm can plug its burrow against temporarily adverse environmental conditions is still a matter of controversy (Hoffman and Mangum, 1972; Vogel and Bretz, 1972).

Table I shows that with the exception of these questionable records for lugworms at 8‰S (23% SW), the lowest limit for worms in the field is considerably higher, from 41% SW (Reid, 1929) to 60–70% SW (Percival, 1929; Popham, 1966; this report). This high value for long-term survival of low salinities in the field is consistent with those few studies on long-term survival in the laboratory, and not inconsistent with observations that lugworms can survive temporary exposure to lower salinities both in the laboratory and in the field.

In the Coos Bay system (Fig. 1), as elsewhere (Hobson, 1967), *Ab. pacifica* is not found in such obviously unsuitable substrates as rocks, gravels, and coarse sand. It is often very abundant in finer sandy-muds with a moderate organic matter content. This distribution pattern seems typical of lugworms generally (*e.g.,* Longbottom, 1970), probably due to the similar feeding and burrowing mechanisms of all members of the family (Hobson, 1967). However, differences in details of substrate preference have been observed (Healy, 1963; Hobson, 1967) such that sympatric species are not usually found together. Sediments suitable for lugworms require calm waters and a depositional environment for development, and while such situations commonly occur in estuaries, lugworms can also be found along the open coast in situations in which wave action is much reduced (Wells, 1963). Thus, *Ab. pacifica* is present on the purely marine beach at Sunset Bay (Fig. 1), where there is almost no wave action and sediments are similar to those within Coos Bay. Suitable muddy-sand substrates are widespread in the Coos Bay system, and *Ab. pacifica* is present, and often abundant, in most such areas (Fig. 1). However, *Ab. pacifica* is absent from certain areas in Coos Bay which seemingly have an appropriate substrate (Fig. 1). There seem to be two major categories of intertidal areas lacking lugworms in Coos Bay: areas subject to severe human disturbance in the form of inputs of domestic and industrial wastes, (*e.g.*, paper pulp mills, log storage areas, commercial shipping docks, fish canneries, *etc.*), and areas receiving freshwater inflows, either year-round or seasonally.

With reference to pollution, of particular interest is the paper mill at mile marker 4 (Fig. 1), which discharges about 1.5 million gallons of wastes each day onto a broad intertidal flat (U. S. Department of the Interior, 1971). These wastes are a complex mixture of partially digested wood residues, including high concentrations of polysaccharides and lignins, and sulfite waste liquors with a variety of acidic sulfur compounds at a pH of 2.0–2.5. Within about 1000 m both upstream and downstream of the outfall plume there is little or no infaunal life on the mudflats. However, *Ab. pacifica* approaches the outfall plume more closely than any other macroscopic invertebrate. In the area most seriously affected by sulfite waste liquor disposal, the only animals present were a few small lugworms. Fecal castings were sometimes observed immediately adjacent to places where H_2S bubbled out of the substrate upon agitation and where sulfur-oxidizing bacteria had formed large mold-like patches of colloidal elemental sulfur on the surface. Several reports indicate that lugworms are notably tolerant of sulfide (Clay, 1959; Hecht, 1932; Perkins and Abbott, 1972; Sveshnikov, 1963). Patel and Spencer (1963) found that blood from *A. marina* contained an active catalyst of sulfide oxidation, an oxidation product of haemoglobin they termed the "brown pigment." Patel and Spencer concluded (page 174) that "The presence of free haemin in the blood, coupled with its autocatalytic production by sulphide must afford the animal considerable protection against the toxic effects of sulphide." In view of the high tolerance of *Ab. pacifica* to sulfide conditions in the outfall plume from this pulp mill, it seems likely that this protective role of the brown pigment is found in all lugworms.

The relationship of the distribution of *Ab. pacifica* to freshwater inflows in the Coos Bay system is shown most obviously by the fact that lugworms are generally absent from the mouths of small streams entering the Bay, such as in South and North Sloughs, Haynes and Kentuck Inlets, and the Sloughs near Cooston (Fig. 1). At their farthest penetration up these streams, lugworms were found only on muddy-sand banks at the sides of the stream channels, exposed to air at most low tides (Porch, 1970). Such worms would not be exposed to the lowest salinities present at such locations over a single tidal cycle (Nicol, 1935; Oglesby, 1969b). Because of their small watersheds, these small streams would not increase much in volume during the rainy season, and thus the lugworms on the side banks would probably not have to contend with much lower salinities during the winter than were observed in the summer.

At the mouth of the Coos River in the Bay (from mile markers 15–17 north to Cooston) there are extensive areas of intertidal mudflats which lacked lugworms. In summer 1970, interstitial salinities here were no lower than 28‰ (80% SW), even though lugworms were encountered elsewhere in the Bay in muds with interstitial salinities as low as 25‰ (71% SW). The Coos River itself has a variable flow, seldom exceeding 100 cubic feet per second during the dry season (May to October), but sometimes discharging as much as 1000 times more water during the rainy season (U. S. Department of the Interior, 1971). This intertidal area near the mouth of the river would be particularly subject to low salinities during periods of high river volume, and such periods may be expected to last many days to several weeks. At such times, interstitial salinities must surely drop to levels far lower than the low-salinity tolerance limits of *Ab. pacifica* (about 50% SW). The fact that *Nereis limnicola* is abundant in the area also suggests that these mudflats are exposed to low salinities for prolonged periods. *N. limnicola* is tolerant of low salinities and fresh water, and is usually restricted by interspecific competition to such habitats (Oglesby, 1965, 1968a; Smith, 1953). While settlement of lugworm larvae may possibly occur on these mudflats during the summer, young worms would be unlikely to survive lengthy low salinity conditions during the winter, and thus permanent populations could not become established.

It seems reasonable to conclude that lugworms are important members of the mudflat infauna only in those estuaries which are sufficiently dominated by the sea that interstitial salinities do not drop below about 50% SW, and where the salinity of the overlying water does not drop below this critical level for more than a few days. Thus, *Ab. pacifica* is one of the most widespread polychaetes in the Coos Bay system, not because it can osmoregulate or even tolerate salinities below about 50% SW, but because most of the Coos Bay system is not exposed to lower salinities.

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SUMMARY

1. The lugworm *Abarenicola pacifica* can tolerate salinities as low as 23% SW in the laboratory, but is unlikely to survive more than brief exposure to salinities lower than 50% SW in the field.

2. Over this salinity range, *Ab. pacifica* is an osmotic conformer, but the coelomic fluids are slightly hyperosmotic to the medium. The literature suggests that other members of the family have a similar osmotic behavior.

3. At all salinities, the coelomic fluids of *Ab. pacifica* are slightly hypo-ionic to the medium with respect to Cl^- and Na^+. Ion regulation appears to be very limited in all members of the family.

4. *Ab. pacifica* has very little ability to control its water content and volume in different salinities.

5. The relationship of the osmotic physiology of lugworms to their distribution in estuaries is discussed. While lugworms may be able to avoid contact with overlying waters of too low salinity for perhaps a few days, they are unlikely to form permanent populations in those parts of estuaries where the interstitial salinities drop below 50% SW during any part of the year.

LITERATURE CITED

ASHWORTH, J. H., 1904. *Arenicola (The Lug Worm). Liverpool Mar. Biol. Comm. Mem.*, **11**: 1–118.

BELYAEV, G. M., 1957. Physiological characteristics of representatives of the same species in waters of different degrees of salinity. *Trudy Vsesoyuznogo Gidrobiologischeskogo Obshchestva (Akad. Nauk SSSR)*, **8**: 321–353. [in Russian]

BIALASZEWICZ, K., 1933. Contribution a l'étude de la composition minérale des liquides nourriciers chez les animaux marins. *Arch. Int. Physiol.*, **36**: 41–53.

BURT, W. V., AND W. B. MCALISTER, 1959. Recent studies in the hydrography of Oregon estuaries. *Res. Briefs, Fish Comm. Oregon*, **7**: 14–27.

BURT, W. V., AND J. QUEEN, 1957. Tidal overmixing in estuaries. *Science*, **126**: 973–974.

CHAPMAN, G., AND G. E. NEWELL, 1947. The rôle of the body fluid in relation to movement in soft-bodied invertebrates. I. The burrowing of *Arenicola. Proc. Roy. Soc. London*, **134B**: 431–455.

CLARK, M. E., 1968a. Free amino-acid levels in the coelomic fluid and body wall of polychaetes. *Biol. Bull.* **134**: 35–47.

CLARK, M. E., 1968b. A survey of the effect of osmotic dilution on free amino acids of various polychaetes. *Biol. Bull.*, **134**: 252–260.

CLAY, E., 1959. Literature survey of the common fauna of estuaries. 2. *Arenicola marina.* [Unpublished, available from the Imperial Chemical Industries Ltd. Brixham, England, 26 pp].

Dakin, W. J., 1908. Variations in the osmotic concentration of the blood and coelomic fluids of aquatic animals, caused by changes in the external medium. *Biochem. J.*, **3**: 473–490.

Darnell, R. M., 1967. Organic detritus in relation to the estuarine ecosystem. Pages 376–382 in G. H. Lauff, Ed., *Estuaries.* American Association Advancement Science Publication 83.

Day, J. H., 1967. The biology of Knynsa Estuary, South Africa. Pages 397–407 in G. H. Lauff, Ed., *Estuaries.* American Association Advancement of Science Publications 83.

Dice, J. F., Jr., 1969. Osmoregulation and salinity tolerance in the polychaete annelid *Cirriformia spirabrancha* (Moore, 1904). *Comp. Biochem. Physiol.*, **28**: 1331–1343.

Duchâteau-Bosson, G., C. Jeuniaux and M. Florkin, 1961. Rôle de la variation de la composante amino-acide intracellulaire dans l'euryhalinité d'*Arenicola marina* L. *Arch. Int. Physiol. Biochim.*, **69**: 30–35.

Florkin, M., 1936. Taux ces substances réductrices et fermentescibles dans les liquides organique de l'arénicale, du dasybranche et du siponcle. *C. R. Soc. Biol. Paris,* **123**: 1022–1024.

Florkin, M., and R. Houet, 1939. Présence d'acide allantoïque dans le liquide coelomique d'annélides et de sipunculiens, et en particulier dans les hématies de ces derniers. *C. R. Soc. Biol. Paris*, **131**: 1276–1277.

Gilles, R., 1972. Osmoregulation in three molluscs: *Acanthochitona discrepans* (Brown), *Glycymeris glycymeris* (L.) and *Mytilus edulis* (L.). *Biol. Bull.*, **142**: 25–35.

Ginetzinsky, A. G., 1959. Two kinds of adaptation of poikilosmotic animals to hypotonic medium. *Proc. Int. Congr. Zool.*, **15**: 958–959.

Green, J., 1968. *The Biology of Estuarine Animals.* Sidgwick & Jackson, London, 401 pp.

Gross, W. J., 1964. Trends in water and salt regulation among aquatic and amphibious crabs. *Biol. Bull.*, **127**: 447–466.

Healy, E. A., 1963. Mucous secretions of abarenicolid lugworms. *Ann. New York Acad. Sci.*, **106**(2): 444–450.

Healy, E. A., and G. P. Wells, 1959. Three new lugworms (Arenicolidae, Polychaeta) from the north Pacific area. *Proc. Zool. Soc. London*, **133**: 315–335.

Hecht, F., 1932. Der chemische Einfluss organischer Zersetzungsstoffe auf das Benthos, dargelegt an Untersuchungen mit marinen Polychaeten, insbesondere *Arenicola marina* L. *Senckenbergiana,* **14**: 199–220.

Hobson, K. D., 1967. The feeding and ecology of two north Pacific *Abarenicola* species (Arenicolidae, Polychaeta). *Biol. Bull.,* **133**: 343–354.

Hoffman, R. J., and C. P. Mangum, 1972. Passive ventilation in benthic annelids? *Science*, **176**: 1356.

Hogue, E. W., and L. C. Oglesby, 1972. Further observations on salt balance in the sipunculid worm *Themiste dyscritum.* *Comp. Biochem. Physiol.,* **42A**: 915–926.

Khlebovich, V. V., 1969. Aspects of animal evolution related to critical salinity and internal state. *Marine Biology,* **2**: 338–345.

Kinne, O., 1971. Salinity. Animals. Invertebrates. Pages 821–995 in O. Kinne, Ed., *Marine Ecology. Volume I. Environmental Factors. Part 2.* Wiley-Interscience, New York.

Klein, G. DeV., 1967. Comparison of recent and ancient tidal flat and estuarine sediments. Pages 207–218 in G. H. Lauff, Ed. *Estuaries.* American Association Advancement of Science Publication 83.

Krogh, A., 1939. *Osmotic Regulation in Aquatic Animals.* Cambridge University Press, Cambridge, 242 pp.

Krüger, F., 1971. Bau und Leben des Wattwurmes *Arenicola marina.* *Helgoländer Wiss. Meeresunters.,* **22**: 149–200.

Longbottom, M. R., 1970. The distribution of *Arenicola marina* (L.) with particular reference to the effects of particle size and organic matter of the sediments. *J. Exp. Mar. Biol Ecol.*, **5**: 138–157.

MacGinitie, G. E., 1939. Some effects of fresh water on the fauna of a marine harbor. *Amer. Midland Natur.*, **21**: 681–686.

Mangum, C. P., and J. M. Shick, 1972. The pH of body fluids of marine invertebrates. *Comp. Biochem. Physiol.,* **42A**: 693–697.

May, D. R., 1972. The effects of oxygen concentration and anoxia on respiration of *Abarenicola pacifica* and *Lumbrineris zonata* (Polychaeta). *Biol. Bull.,* **142**: 71–83

NESTEROV, V. P., AND I. A. SKULSKI, 1965. Comparative investigations of Li, Na, K, Rb, and Ca distribution in muscle tissue of marine animals. *Zh. Evol. Biokhim. Fizol.*, **1**: 151–157. [in Russian]

NEWMAN, W. A., 1967. On physiology and behavior of estuarine barnacles. *Proc. Symp. Crustacea, Enakulam, India, Part III*, **1967**: 1038–1066.

NICOL, E. A. T., 1935. The ecology of a salt-marsh. *J. Mar. Biol. Ass. U. K.*, **20**: 203–261.

OGLESBY, L. C., 1965. Steady-state parameters of water and chloride regulation in estuarine nereid polychaetes. *Comp. Biochem. Physiol.*, **14**: 621–640.

OGLESBY, L. C., 1968a. Responses of an estuarine population of the polychaete *Nereis limincola* to osmotic stress. *Biol. Bull.*, **134**: 118–138.

OGLESBY, L. C., 1968b. Some osmotic responses of the sipunculid worm *Themiste dyscritum*. *Comp. Biochem. Physiol.*, **26**: 155–177.

OGLESBY, L. C., 1969a. Inorganic components and metabolism; ionic and osmotic regulation: Annelida, Sipuncula, Echiura. Pages 211–310 in M. Florkin and B. T. Scheer, Eds., *Chemical Zoology*, IV. Academic Press, New York.

OGLESBY, L. C., 1969b. Salinity-stress and desiccation in intertidal worms. *Amer. Zool.*, **9**: 319–331.

OGLESBY, L. C., 1970. Studies on the salt and water balance of *Nereis diversicolor*. I. Steady-state parameters. *Comp. Biochem. Physiol.*, **36**: 449–466.

OGLESBY, L. C., 1973. An analysis of water-content regulation in selected worms. In press in F. J. Vernberg, Ed., *Proceedings of Symposium on the Physiological Ecology of Estuarine Organisms*. University of South Carolina, Columbia, South Carolina.

ORTON, J. H., 1925. Experimental regeneration in the sea of the hind body of *Arenicola marina* L. *Ann. Mag. Nat. Hist.* **16**: 641–643.

PATEL, S., AND C. P. SPENCER, 1963. The oxidation of sulphide by the haem compounds from the blood of *Arenicola marina*. *J. Mar. Biol. Ass. U. K.*, **43**: 167–175.

PEARSE, A. S., 1928. On the ability of certain marine invertebrates to live in diluted sea water. *Biol. Bull.*, **54**: 405–409.

PERCIVAL, E., 1929. A report on the fauna of the estuaries of the River Tamar and the River Lynher. *J. Mar. Biol. Ass. U. K.*, **16**: 81–108.

PERKINS, E. J., AND O. J. ABBOTT, 1972. Nutrient enrichment and sand flat fauna. *Marine Pollution Bull.*, **3**(5): 70–72.

PIERCE, S. K., JR., 1970. The water balance of *Modiolus* (Mollusca: Bivalvia: Mytilidae): osmotic concentrations in changing salinities. *Comp. Biochem. Physiol.*, **36**: 521–533.

POPHAM, E. J., 1966. The littoral fauna of the Ribble estuary, Lancashire, England. *Oikos*, **17**: 19–32.

PORCH, L. L., 1970. Polychaetes of Coos Bay. Unpublished student report, 21 pp. [Available from the Institute of Marine Biology, University of Oregon, Charleston, Oregon, 97420.]

POTTS, W. T. W., AND G. PARRY, 1964. *Osmotic and Ionic Regulation in Animals*. Pergamon Press, London, 423 pp.

QUINTON, R., 1900. Communication osmotique, chez l'invertébré marin normal, entre le milieu intérieur de l'animal et le milieu extérieur. *C. R. Acad. Sci. Paris*, **131**: 905–908.

REID, D. M., 1929. On some factors limiting the habitat of *Arenicola marina*. *J. Mar. Biol. Ass. U.K.*, **16**: 109–116.

REMMERT, H., 1969. Über Poikilosmotie und Isoosmotie. *Z. Vergl. Physiol.*, **65**: 421–427.

RHOADS, D. C., 1967. Biogenic reworking of intertidal and subtidal sediments in Barnstable Harbor and Buzzards Bay, Massachusetts. *J. Geol.*, **75**: 461–476.

ROBERTSON, J. D., 1949. Ionic regulation in some marine invertebrates. *J. Exp. Biol.*, **26**: 182–200.

SCHLIEPER, C., 1929. Über die Einwirkung niederer Salzkonzentrationen auf marine Organismen. *Z. Vergl. Physiol.*, **9**: 478–514.

SMITH, R. I., 1953. The distribution of the polychaete *Neanthes lighti* in the Salinas River estuary, California, in relation to salinity, 1948–1952. *Biol. Bull.*, **105**: 335–347.

SMITH, R. I., 1970. Hypo-osmotic urine in *Nereis diversicolor*. *J. Exp. Biol.*, **53**: 101–108.

STRUNK, C., 1930a. Beiträge zur Excretions-Physiologie der Polychäten *Arenicola marina* und *Stylarioides plumosus*. *Zool. Jahrb. Allg. Zool. Physiol. Tiere*, **47**: 259–290.

Strunk, C., 1930b. Excretionsphysiologische Studien an den Polychäten *Polymnia nebulosa* und *Lanice conchilega*. Ein weiterer Beitrag zur Excretionsphysiologie der Polychäten. *Zool. Jahrb. Allg. Zool. Physiol. Tiere*, **48**: 385–398.

Strunk, C., 1932. Über das Vorhandensein und die Aufgaben des Harnstoffs in der Leibeshöhlenflüssigkeit des Polychäten *Arenicola marina*. *Zool. Jahrb. Allg. Zool. Physiol. Tiere*, **52**: 216–222.

Sutcliffe, D. W., 1967. Sodium regulation in the amphipod *Gammarus duebeni* from brackish-water and fresh-water localities in Britain. *J. Exp. Biol.*, **46**: 529–550.

Sveshnikov, V. A., 1963. The biocoenotic associations and living conditions of some food invertebrates of the littoral of the Gulf of Kandalaksha in the White Sea. *Trudy Kandalakshsk. Gos. Zapovednika*, **4**: 114–134. [in Russian]

United States Department of the Interior, 1971. *Natural Resources, Ecological Aspects, Uses, and Guidelines for the Management of Coos Bay, Oregon. A Special Report*, 128 pp. [Available from the Secretary, Pacific Northwest Region, U. S. Department of the Interior, P. O. Box 3621, Portland, Oregon 97208].

Vogel, S., and W. L. Bretz, 1972. Passive ventilation in benthic annelids? *Science*, **176**: 1356–1357.

Wells, G. P., 1945. The mode of life of *Arenicola marina* L. *J. Mar. Biol. Ass. U.K.*, **26**: 170–207.

Wells, G. P., 1959. The genera of Arencolidae (Polychaeta). *Proc. Zool. Soc. London*, **133**: 301–314.

Wells, G. P., 1963. Barriers and speciation in lugworms (Arenicolidae, Polychaeta). Pages 79–98 in J. P. Harding and N. Tebble, Eds., *Speciation in the Sea*. Systematics Association Publication 5, London.

Wells, G. P., and I. C. Ledingham, 1940. Physiological effects of a hypotonic environment. I. The action of hypotonic salines on isolated rhythmic preparations from polychaete worms (*Arenicola marina, Nereis diversicolor, Perinereis cultrifera*). *J. Exp. Biol.*, **17**: 337–352.

Zenkevich, L. A., 1938a. The influence of Caspian and Black Sea waters of different concentrations upon some common Black Sea invertebrates (to the question of the acclimatization of Black Sea invertebrates in the Caspian Sea). Part I. Survivorship and body weight changes. *Zool. Zh.*, **17**: 845–876. [in Russian]

Zenkevich, L. A., 1938b. The influence of Caspian and Black Sea waters of different concentrations upon some common Black Sea invertebrates (to the question of the acclimatization of Black Sea invertebrates in the Caspian Sea). Part II. (The change of internal salinity). *Zool. Zh.*, **17**: 976–1002. [in Russian]

Reference: *Biol. Bull.*, **145**: 200–209. (August, 1973)

CAUSES OF DAILY RHYTHMS IN PHOTOSYNTHETIC RATES OF PHYTOPLANKTON

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Rhythmic rates are characteristic of several processes in the phytoplankton. Daily oscillations are known for rates of photosynthesis (Doty and Oguri, 1957; Verduin, 1957), of chlorophyll synthesis (Yentsch and Ryther, 1957; Shimada, 1958), and rates of nutrient uptake (Goering, Dugdale and Menzel, 1964). Since the initial discovery, a number of generalizations have encouraged belief that most phytoplankton communities are rhythmic. The daily maximum in photosynthetic rate is recorded for early morning in the ocean near the equator (Doty and Oguri, 1957), for later in the day in lakes (Lorenzen, 1963), and in inshore marine environments (Newhouse, Doty and Tsuda, 1967). There is also considerable evidence that the amplitude of the daily oscillations decreases with increase in latitude (Doty, 1959).

Two categories of explanations for photosynthetic rhythms are extant in the literature, neither resolved. The first, which may be called the phasing hypothesis, is based on an intrinsic characteristic of algae, namely the ability to have cell processes entrained with a light-dark cycle. The alternative or "forcing" hypothesis is that some time dependent deficiency (nutrients) or destructive action (*e.g.*, photo-destruction) causes the oscillation.

A most cogent argument for the forcing hypothesis is based on the daily oscillation in concentrations of critical nutrients. This argument proposes that the rate of nutrient uptake is directly a function of external concentration of a rate limiting nutrient as described by Michaelis-Menten kinetics (Dugdale, 1967). Rates of uptake and growth in chemostats are offered in support (Caperon, 1967; Eppley and Coatsworth, 1968; Eppley and Thomas, 1969). Under such conditions the growth response at any concentration of nutrient would be invariant with time. Daily oscillations in photosynthesis could result, for example, from daily oscillations in nitrate and ammonium ions (Goering, Dugdale and Menzel, 1964).

The same kinetics popularized by Dugdale (1967) may be used to construct models of daily rhythms in photosynthetic potential (U_{max}). In one model the rhythm is phased to the daily rhythm of environment. In the other it is forced (Fig. 1). In the model of phased oscillation, the kinetic constants of the hyperbola oscillate (Fig. 1A). In the model of a forced oscillation, only the external concentration of a limiting nutrient oscillates (Fig. 1B).

Further analysis is possible with the models. If the phytoplankton is nutrient limited and the concentration of nutrient(s) is constant or oscillatory but with a maximum in the morning, as is characteristic, one could expect the largest response to added nutrient at some phase of the daily cycle. The timing of the maximum response to enrichment may also be expected to differ in the case of the alternative models. The largest response to added nutrient ought to occur at the time of the daily maximum in U_{max} if U_{max} oscillates. Conversely, the largest response should be at the time of the daily minimum if the daily rhythm is caused only by an oscillation in external nutrient. Two reference points are provided in each experimental verification. One is the so-called photosynthetic capacity which is the rate of photosynthesis at light saturation or P_{max}. The second is the photosynthetic potential which is the rate at both light and nutrient saturation or U_{max}.

The first model (Fig. 1A) describes an intrinsic oscillation in the potential for photosynthesis U_{max} as shown for two time points in the daily cycle. To resolve U_{max} additional nutrient, a, is added to the existing or native (n) concentration which is constant in the model. Actual photosynthetic capacity, U, is shown as two horizontal, dashed lines. At one time U is increased by "p" amount and at another by "t" amount. Note that the daily amplitude of U_{max} is greater than for U. The half-saturation constant, K_t, was held constant although it too may oscillate thereby changing the value of "p" or "t." Clearly, the intrinsic oscillation is in potential. The daily oscillation in photosynthetic capacity may be some combination of intrinsic and forced.

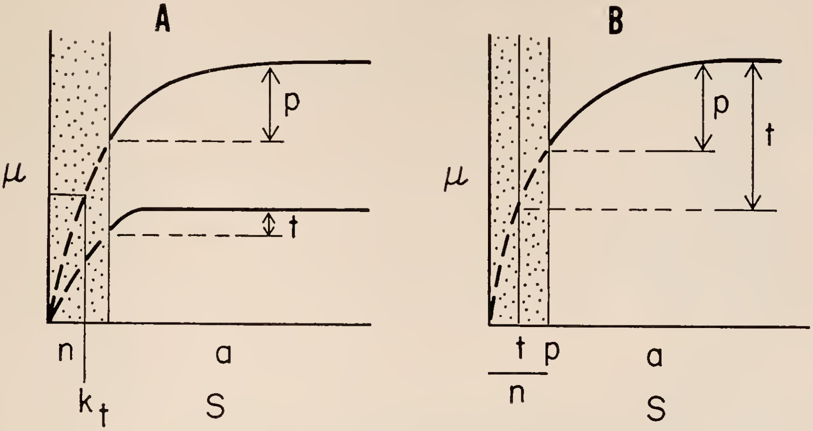

FIGURE 1. Alternative conditions permitting a diurnal oscillation in rates of phytoplankton photosynthesis. In both conditions the rate of carbon assimilation (photosynthesis) U is proportional to the external concentration of the rate limiting nutrient, S. The first condition postulates an intrinsic oscillation in U_{max} while the native (n) concentration of nutrient remains constant. The second condition postulates a constant U_{max} while the concentration of limiting nutrient forces photosynthetic rate to oscillate. See text for explantion.

The second model (Fig. 1B) describes a forced oscillation in photosynthetic capacity, forced solely by an oscillation in external nutrient. The potential for photosynthesis, U_{max}, remains constant. Two points on the daily oscillation of capacity are shown by the dashed horizontal lines, the result of native nutrient, n, being at concentrations "t" and "p," respectively. Note that the addition of nutrient to resolve the hyperbola results in an increase in U by "t" and "p" amounts, respectively, and the amount of stimulation is greater at time "t" (trough) than at time "p" (peak) or the reverse of that seen in the intrinsic oscillation. In other words two techniques are suggested for distinguishing intrinsic from forced daily rhythms in photosynthetic rates. One criterion is the behavior of U_{max} and the second is the time of day in which response to enrichment is maximum.

METHODS

Rates of photosynthesis (U) were measured as rates of carbon assimilation in a standard 3-hour incubation with ^{14}C labeled bicarbonate technique (Steemann-Nielsen, 1952). Rates, corrected for excretory loss, were measured in unenriched samples and in samples to which six concentrations of sodium (primary) phosphate ranging from 0.25 to 4.0 micromoles P/liter had been added. Each experiment was repeated at seven different times within a 24 or 28-hour interval. One set of measurements was carried out beginning August 6 and a second on October 16, 1970.

TABLE I

Nutrients employed to enrich water from Lake George in the second experiment

	Milligrams/liter
$NH_4\ NO_3$	2.0
$Mg\ SO_4$	5.0
$Ca\ Cl_2 \cdot 2H_2O$	3.68
KCl	0.95
Fe (as $Fe\ Cl_3$)	0.20
$Na_2\ SiO_3 \cdot 9H_2O$	1.25
Trace Elements (B, Co, Cu, Mn, Mo, Zn)	*
Vitamins	
B_1	0.0025
B_{12}	0.0001
Biotin	0.00005

* Trace elements in nanomoles/liter are H_3BO_4, 14.7, $CoNO_3 \cdot 6H_2O$, 0.7; $CuSO_4 \cdot 5H_2O$, 0.1; $McCl_2 \cdot 4H_2O$, 1.6; $H_2MO\ O_4\ H_2O$ (85%) 0.04; and $Zn\ SO_4 \cdot 7H_2O$, 0.3 nanomoles/liter.

Water for the experiments was collected from Lake George, New York (IBP-Station 1; 2 meters depth), filtered through a #25 mesh net (aperture = 64 microns), and stored in glass carboys at the lake surface but shielded from direct sunlight. The samples were incubated on a revolving drum under fluorescent light (cool white) at a saturating intensity (1500 ft-c) and at the temperature of the epilimnion (24.0° C in August and 16.0° C in October). In the second experiment replication was increased from two to three and a "complete" set of nutrients (Table I) was included as an extra treatment.

Kinetic coefficients (U_{max} and k_t) as described by the Michaelis-Menten equation were employed to characterize the growth responses, since uptake rates in both single species cultures (Eppley and Thomas, 1969) and phytoplankton assemblages (MacIsaac and Dugdale, 1968) have been shown to respond hyperbolically to the concentrations of a limiting nutrient. The linear transformation $U = U_{max} - k_t (U/S)$ was used to calculate U_{max} and k_t as recommended by Dowd and Riggs (1965) for unweighted data. Confidence limits (95 per cent) were calculated for

k_t (slope) and U_{max} (intercept). Standard errors were calculated for photosynthetic capacity which was measured in duplicate (experiment 1) or triplicate (experiment 2). The native concentration of phosphate, normally at the lower limit of detection in Lake George, was not measured. It was assumed to be 10 per cent of total phosphorus in suspension which, in August, was approximately 5.0 up P/liter (Clesceri, personal communication). The 10 per cent estimate is consistent with the estimated fraction of reactive phosphorus for many lakes (Hutchinson, 1957).

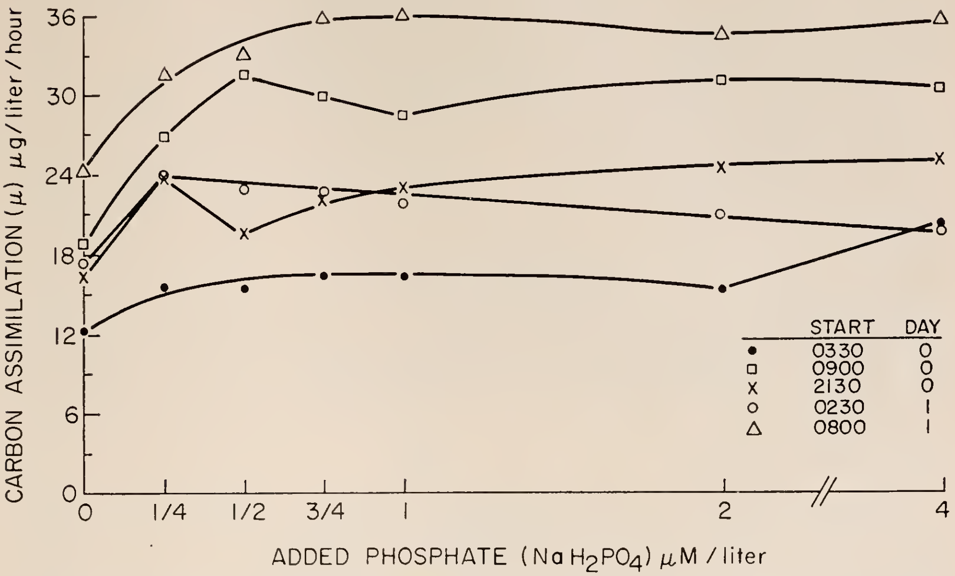

FIGURE 2. Rates of carbon assimilation in response to the addition of sodium phosphate to water samples from Lake George, New York. Shown are the results of five of seven sets of samples measured during one 30-hour interval in October 1970. Incubation was at lake temperature (16°) and at 1500 ft-c.

RESULTS

The addition of phosphate to samples of water from Lake George often but not always resulted in stimulation of carbon assimilation. In one experiment rates of carbon assimilation in five of seven sets of enrichments are shown in Figure 2. Although only a part of the total pattern can be observed, that which is the result of added phosphate is strongly suggestive of an hyperbola. Despite the general conformation, intermediate concentrations of enrichment sometimes produced slight but significant depressions in expected pattern. These so-called stalls were evident in at least two of the seven sets that comprised each experiment. While not confounding, they remind the investigator that the response of many distinct species populations is measured together.

The photosynthetic capacity, *i.e.*, P_{max} (photosynthetic rate at light saturation), of the phytoplankton in Lake George undergoes a characteristic daily oscillation.

In the first experiment (August 6) rates of carbon uptake in the unenriched samples ranged from 13.9 μg C/liter/hr at 0900 EST to 6.6 μg C/liter/hr at 2000 (Fig. 3A). Clearly the phytoplankton was most responsive to enrichment at 0900, the time of the morning maximum and least responsive at the time of the evening minimum in photosynthetic capacity. The rates were increased by 29 and 18 per cent, respectively.

Phosphate depletion in the stored sample could account for the pattern on the second morning of confinement. The oscillation in photosynthetic capacity failed to return to the same rate measured on the morning preceding (Fig. 3A). The potential or U_{max} for photosynthesis was essentially the same as the morning preceding, however, indicating that nutrients and not photosynthesizers were lacking. The rate was increased by 54 per cent on the second morning.

Photosynthetic capacities and photosynthetic potentials again oscillated in experiment 2 (October). The response was similar in essence to the first experiment although it differed in detail (Fig. 3B). Photosynthetic capacity showed a net gain of 37 per cent over a 24-hour interval. At the same time the rates of photosynthesis were much more stimulated by the addition of phosphate. The degree of stimulation was again largest at the time of the morning maximum. Photosynthesis was increased by 62.0 per cent at the morning maximum and by 27.0 per cent at the afternoon minimum. The oscillations appeared to be more irregular and photosynthetic potential showed a secondary peak in the early night. Both experiments reported here yielded the same result which in effect was that phosphate was stimulatory at all times during the experiment and thereby judged to be deficient.

Half-saturation constants

Absolute values for the half-saturation constants are unnecessary to support the model since the growth maximum is essentially independent of it. It is instructive to examine the half-saturation constants (k_t) which have been approximated with the assumption that 10 per cent of total phosphorus in Lake George water is as inorganic phosphate. Estimates of k_t in the first experiment ranged from 0.02 to 0.23 μg P/liter and in the second experiment from 0.12 to 0.30 μg P/liter. They were roughly in the range of saturation constants found for environments of inorganic nitrogen compounds in the infertile areas of the oceans (MacIsaac and Dugdale, 1968); doubling the amount of inorganic phosphate available would only double the estimates of k_t. Within each experiment the estimated constants were different. In most instances the calculated values are below the limit of resolution for phosphate in natural waters and kinetic bioassay such as proposed for glucose (Hobbie and Wright, 1965) may be appropriate.

Limiting nutrient

To reduce the possibility that the addition of phosphate was eliciting a nonnutritive response, a complete set of nutrients (Table I) was included as an additional treatment in the second experiment. The treatment was divided into two parts, one receiving all nutrients except phosphate, the second all nutrients. The experimental result was negative since at none of the seven intervals tested was the growth stimulation in response to all nutrients greater than that to phosphate only.

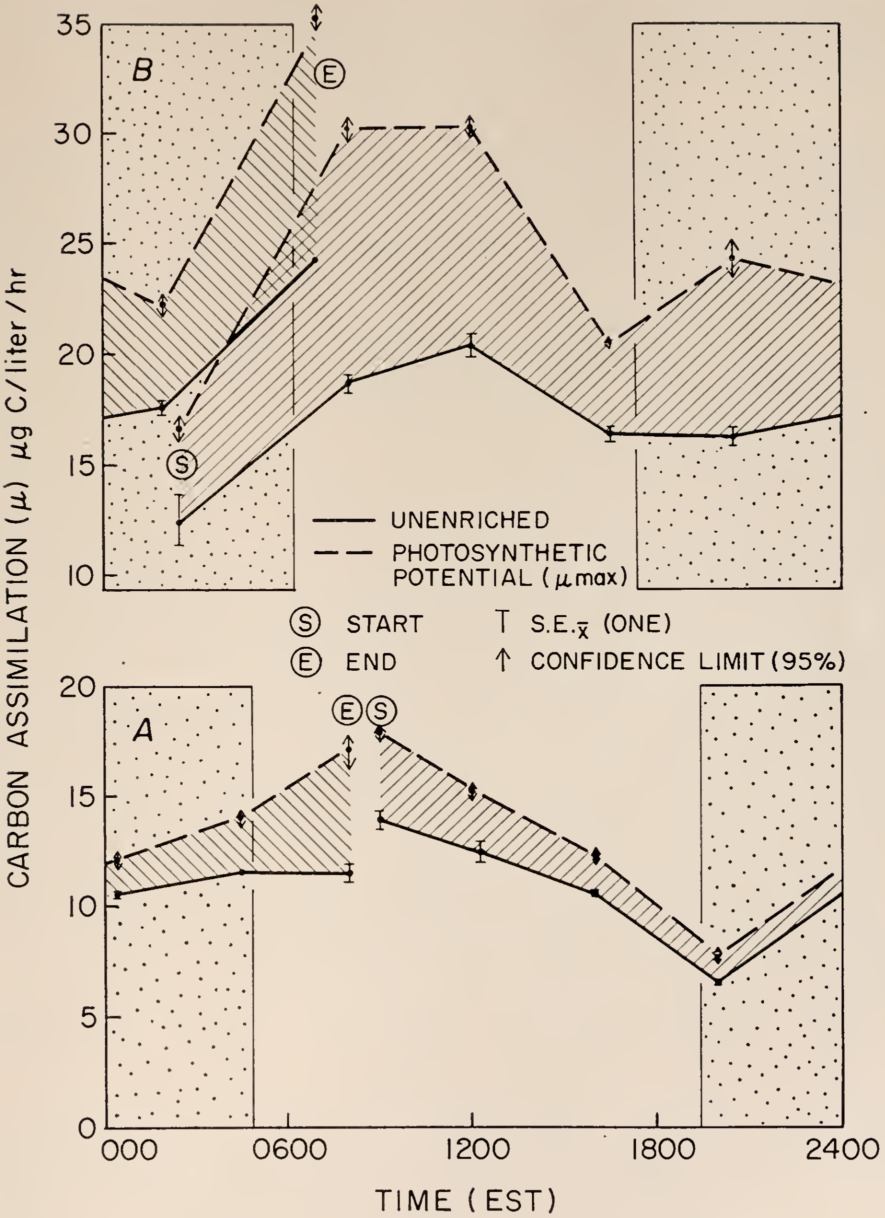

FIGURE 3. Daily rhythms in degree of stimulation achieved by the addition of phosphate to an assemblage of planktonic algae. Boundaries of the polygons were formed by rates of carbon assimilation without addition of phosphate (lower line) and a maximum rate of assimilation or U_{max} (upper boundary). Experiments performed on the phytoplankton of Lake George, New York in August (A) and October (B) 1970.

There was some stimulation in the complete minus phosphate treatment. The level of stimulation was always intermediate to that achieved with phosphate only and could indicate phosphate contamination in the other reagents.

DISCUSSION

Photosynthetic rates of phytoplankton may oscillate each day as a result of a changing photosynthetic potential (U_{max}), or as seemed to be the case in Lake George, the rhythm may be the result of some combination of an intrinsic oscillation in potential and an oscillation in external nutrient concentration. Although the latter was unmeasured in Lake George, much evidence for a daily oscillation exists in the literature. An intrinsic basis is clearly indicated in the data, although the model itself cannot discriminate between an intrinsic rhythmicity in photosynthetic rate and the presence of a second non-nutrient forcing oscillation in the environment. The former may be the more plausible explanation. At least one dominant population, if entrained to the daily cycle, could account for a phased oscillation in photosynthetic potential. Oscillating photosynthetic capacities are characteristic of synchronous cultures (Senger, 1970) as well as cultures of algae entrained but not necessarily all dividing during the permissive or gate phase of each 24-hour cycle, *i.e.*, rhythmic (Sweeney and Hastings, 1958; Bruce, 1970). The mechanism of enrichment although light dependent is not entirely intensity dependent (Senger and Bishop, 1969). The blue-green wavelengths are the most effective at entraining cell cycles, and they are likely to be the most penetrating in clear lakes such as the lake (Lake George) on which the experiments were carried out.

Ample direct evidence for entrained cell cycles of algae in nature exists. Direct observation of dividing cells (Staley, 1971) and the oscillation in density of algal cells in the downstream drift (Müller-Haeckel, 1970) show entrained cell division cycles. An increase in the density of algal cells in suspension results apparently from loss of attachment at the time of cell division as occurs with bacteria in the field (Bott and Brock, 1970) and in culture (Helmstetter and Cummings, 1964). Cell cycles of planktonic species are also known to be entrained in nature (Eppley, Holm-Hansen and Stickland, 1968) although much of the recent evidence is less direct and in the form of activities other than cell division, *e.g.*, enzyme activity (Eppley, Packard and MacIsaac, 1970; Eppley, Rogers, McCarthy and Sournia, 1971).

There is evidence that individual rhythms of cellular activity retain a strict phase relationship (McMurry and Hastings, 1972) such that the phase of one reflects the phase of another. The potential significance of entrained cell cycles in the development of resource acquisition strategies awaits an appropriate "process" model. Eppley (1971) has already indicated the need to know the endogenous oscillation in growth (and nutrient uptake) constants (*e.g.*, U_{max} and k_t) and in the environmental oscillation in concentration of the limiting nutrient. Treatment of an entire assemblage is likely to be complicated by temporally stratified populations (phase separated rhythms of uptake) and a varying degree of coupling between carbon assimilation and nutrient uptake.

One objective of such a model may be to predict the latitudinal variation in phase and amplitude of the diurnal photosynthetic rhythm observed in the phytoplankton (Doty, 1959). In tropical waters the morning maximum was reported to be six or more times larger than the evening minimum, whereas at high latitude it was less than two. Conceivably a model would be based on both intrinsically mediated rhythms and on forced or environmentally dictated rhythms. The consequences of shifting the phase relationship of oscillators internal and external to the alga cell are immediately obvious. If for example the uptake of a limiting nutrient and photosynthetic fixation of carbon are oscillatory with the former driving the latter, phase of the two would determine the amplitude of the daily rhythm in photosynthetic capacity. The amplitude would be progressively damped if the photosynthetic maximum was shifted to later in the day while the nutrient uptake maximum remained in the early morning.

Conceivably, the latitudinal pattern, *i.e.*, the loss of amplitude and apparent shift of the maximum in photosynthetic capacity to later in the day, is due in part to such a mechanism. Doty and Oguri (1957) discovered the maximum to be at dawn in tropical areas. Lorenzen (1963) and Newhouse *et al*. (1967) found late morning to midday maxima at temperate latitudes. Phytoplankton in temperate latitudes may be most responsive to nutrient addition (this paper) and to thermal stimulation (Morgan and Stross, 1969) at midmorning or midday. However, we found arctic phytoplankton to be most responsive to thermal pulsing in late afternoon and evening (unpublished). If indeed the phasing of endogenous rhythms within the cells of algal populations can account for amplitude and phase changes with latitude, there is reason to expect other insights from a model.

In the simple analysis above the assemblage is viewed as a single species when an assemblage of many species exists reach conceivably with a unique rhythm. Coexistence of potential competitors gives statistical evidence that competition is avoided (Hutchinson, 1961). Unique phase and amplitude characteristics of growth (Hastings and Sweeney, 1964) and uptake kinetics of co-dominant species have been suggested as one mechanism for avoiding or minimizing competition in an environment where nutrient resource inputs are continuous (Williams, 1971; Eppley *et al.* 1971). Conceivably temporal uniqueness in nutrient uptake is a feature of the co-dominant populations in a phytoplankton assemblage. A model containing sufficient detail of the endogenous characteristics of co-dominant populations, if viewed over a wide range in photoperiods (or latitude), may well provide insight into the significance of endogenous rhythms. For the moment at least it is sufficient to suspect that field rhythms are a compromise between a forcing oscillation in the environment and an inherent rhythmicity in potential of the organisms (Enright, 1970).

Field experiments conducted at the lakeside laboratory of Freshwater Institute of Rensselaer Polytechnic Institute. Work supported in part by USIBP EDFB with funds from NSF through AEC: AG-199, 40–193–69.

SUMMARY

The cause for the daily rhythm in the photosynthetic capacity of phytoplankton has been examined. Alternative hypotheses have been modeled with the Michaelis-Menten equation. In one model rates of photosynthesis oscillate in response to a forcing from the external concentration of limiting nutrient while photosynthetic

potential (U_{max}) of the assemblage remains constant. In the alternative model photosynthetic potential oscillates in response to intrinsic organization of the cell. The alternatives may be deduced from the photosynthetic response to added nutrient.

The photosynthetic response to added phosphate was tested with water from Lake George, New York. Rates of carbon assimilation in the unenriched controls described a daily oscillation with an amplitude of approximately two and a phase maximum in midmorning. The degree of stimulation to added phosphate was also rhythmic. The maximum and minimum corresponded with the daily maximum and minimum, respectively, in unenriched controls. The oscillating intensity of response was interpreted as a changing potential in the intrinsic capacity of the algal assemblage.

The display of intrinsic rhythms by at least one dominant component in the algal assemblage infers the entrainment of activity cycles to the daily cycle. Additional evidence to support the inference is described. Although the algae may be entrained, there is also evidence from the literature that nutrient concentrations undergo daily oscillations. Photosynthetic rhythms could result from both an intrinsic and a nutrient (forcing) oscillation. A changing phase relationship between the two could explain the decline in amplitude of the photosynthetic rhythm with increase in latitude.

LITERATURE CITED

BOTT, T. L., AND T. D. BROCK, 1970. Growth and metabolism of periphytic bacteria: methodology. *Limnol. Oceanogr.*, **15**: 333–342.

BRUCE, V. G., 1970. The biological clock in *Chlamydomonas reinhardi*. *J. Protozool.*, **17**: 328–334.

CAPERON, J., 1967. Population growth in micro-organisms limited by food supply. *Ecology*, **48**: 715–722.

DOTY, M. S., 1959. Phytoplankton photosynthetic periodicity as a function of latitude. *J. Mar. Biol. Ass. India*, **1**: 66–68.

DOTY, M. S., AND M. OGURI, 1957. Evidence for a photosynthetic daily periodicity. *Limnol. Oceanogr.*, **2**: 37–40.

DOWD, J. E., AND D. S. RIGGS, 1965. A comparison of estimates of Michaelis-Menten kinetic constants from various linear transformations. *J. Biol. Chem.*, **246**: 863–869.

DUGDALE, R. C., 1967. Nutrient limitation in the sea: dynamics, identification and significance. *Limnol. Oceanogr.*, **12**: 685–695.

ENRIGHT, J. T., 1970. Ecological aspects of endogenous rhythmicity. Pages 221–238 in R. F. Johnston, Ed., *Annual Review of Ecology and Systematics, 1970.* Annual Reviews, Palo Alto, California.

EPPLEY, R. W., AND J. L. COATSWORTH, 1968. Nitrate and nitrite uptakes by *Ditylum brightwellii:* kinetics and mechanisms. *J. Phycology*, **4**: 151–156.

EPPLEY, R. W., AND W. H. THOMAS, 1969. Comparison of half-saturation constants for growth and nitrate uptake of marine phytoplankton. *J. Phycology*, **5**: 375–379.

EPPLEY, R. W., O. HOLM-HANSEN AND J. D. H. STRICKLAND, 1968. Some observations on the vertical migration of dinoflagellates. *J. Phycology*, **4**: 333–340.

EPPLEY, R. W., T. T. PACKARD AND J. J. MACISSAC, 1970. Nitrate reductase in Peru Current phytoplankton. *Mar. Biol.* **6**: 195–199.

EPPLEY, R. W., J. N. ROGERS, J. J. MCCARTHY AND A. SOURNIA, 1971. Light/dark periodicity in nitrogen assimilation of the marine phytoplankters *Skeletonema costatum* and *Coccolithus huxleyi* in N-limited chemostat culture. *J. Phycology*, **7**: 150–154.

GOERING, J. J., R. C. DUGDALE AND D. W. MENZEL, 1964. Cyclic diurnal variations in the uptake of ammonia and nitrate by photosynthetic organisms in the Sargasso Sea. *Limnol. Oceanogr.*, **9**: 448–451.

HASTINGS, J. W., AND B. M. SWEENEY, 1964. Phased cell division in the marine dinoflagellates. Pages 307–321 in E. Zeuthen, Ed., *Synchrony of Cell Division and Growth*. J. Wiley and Sons, New York.

HELMSTETTER, C. E., AND D. J. CUMMINGS, 1964. An improved method for the selection of bacterial cells at division. *Biochim. Biophys. Acta,* **82**: 608–610.

HOBBIE, J. E., AND R. T. WRIGHT, 1965. Bioassay with bacterial uptake kinetics: glucose in freshwater. *Limnol. Oceanogr.,* **10**: 471–474.

HUTCHINSON, G. E., 1957. *A Treatise on Limnology, Vol. I. Geography, Physics and Chemistry*. J. Wiley and Sons, New York, 1015 pp.

HUTCHINSON, G. E., 1961. The paradox of the plankton. *Amer. Natur.,* **95**: 137–145.

LORENZEN, C. J., 1963. Diurnal variation in photosynthetic activity of natural phytoplankton populations. *Limnol. Oceanogr.,* **8**: 56–62.

MACISAAC, J. J., AND R. C. DUGDALE, 1968. The kinetics of nitrate and ammonia uptake by natural populations of marine phytoplankton. *Deep Sea Res.,* **16**: 415–422.

MCMURRY, L., AND J. W. HASTINGS, 1972. No desynchronization among four circadian rhythms in the unicellular alga, *Gonyaulax polyedra. Science,* **175**: 1137–8.

MORGAN, R. P., AND R. G. STROSS, 1969. Destruction of phytoplankton in the cooling water supply of a steam electric station. *Chesapeake Sci.,* **10**: 165–171.

MÜLLER-HAECKEL, A., 1970. Wechsel der Aktivitätsphase bei einzelligen Algen fliessender Gewasser. *Oikos,* (Supp) **13**: 134–138.

NEWHOUSE, J., M. S. DOTY AND R. T. TSUDA, 1967. Some diurnal features of a neritic surface plankton population. *Limnol. Oceanogr.,* **12**: 207–212.

SENGER, H., 1970. Charakterisierung einer synchronkultur von *Scenedesmus obliquus,* ihren potentiellen Photosyntheseleistung und des Photosynthesese-Quotienten während der Entwicklungscyclus. *Planta Med.,* **90**: 243–266.

SENGER, H., AND N. I. BISHOP, 1969. Light-dependent formation of nucleic acids and its relation to the induction of synchronous cell division in *Chlorella*. Pages 179–202 in G. M. Padilla, G. L. Whitson and I. L. Cameron, Eds., *The Cell Cycle*. Academic Press, New York.

SHIMADA, B. M., 1958. Diurnal fluctuations in photosynthetic rate and cholorphyll "a" content of phytoplankton from Eastern Pacific Waters. *Limmnol. Oceanogr.,* **3**: 336–339.

STALEY, J. T., 1971. Growth rates of algae determined *in situ* using an immersed microscope. *J. Phycology,* **7**: 13–17.

STEEMANN-NIELSEN, E., 1952. The use of radioactive carbon (^{14}C) for measuring organic production in the sea. *J. Cons. Exp. Mer.,* **18**: 117–140.

SWEENEY, B. M., AND J. W. HASTINGS, 1958. Rhythmic cell division in populations of *Gonyaulax polyedra. J. Protozool.,* **5**: 217–224.

VERDUIN, J., 1957. Daytime variations in phytoplankton. *Limnol. Oceanogr.,* **2**: 333–336.

WILLIAMS, F. M., 1971. Dynamics of microbial populations. Pages 197–267 in B. C. Patten, Ed., *Sytems Analysis and Simulation in Ecology,* Vol. I. Academic Press, New York.

YENTSCH, C. S., AND J. H. RYTHER, 1957. Short-term variations in phytoplankton chlorophyll and their significance. *Limnol. Oceanogr.,* **2**: 140–142.

Reference: *Biol. Bull.*, **145**: 210–222. (August, 1973)

THE EFFECT OF ENVIRONMENTAL FACTORS ON THE GROWTH OF A HALOPHYLIC SPECIES OF ALGAE

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True obligate halophylic algae will only grow well at elevated salt concentrations. These species have adapted very well to a severe environmental situation. An important question to ask about these halophytes is how they are able to survive such high salt concentrations, and what is the effect of various ecological parameters on their growth rates. The purpose of this study was to define the environmental factors limiting the growth of this species, and to see how these factors could be manipulated in order to produce high yields of cells for physiological and biochemical investigations.

Smith (1950) reported that the genus *Dunaliella* is worldwide in distribution and is almost invariably present in salterns and brine lakes. Butcher (1959) included 12 species in this genus, and stated they occur in diverse habitats including any situation from almost fresh water to salt brine. Many studies have been carried out on a variety of halophytes of the genus *Dunaliella* and *Chlamydomonas*. These studies have included, to name a few areas, culture methods and ecological parameters (Kirkpatrick, 1934; McLachlan, 1960; Yamada and Okamoto, 1961), sodium requirement (McLachlan, 1960), reproduction (Latorella, 1971), salt tolerance and osmoregulation (Baas-Becking, 1931; Marrè and Servettaz, 1959; Johnson, Johnson, MacElroy, Speer and Bruff, 1968; Okamoto and Suzuki, 1964; Yamamoto, 1967; Yamamoto and Okamoto, 1967, 1968; Wegmann, 1971), membrane characteristics (Jokela, 1969), photosynthesis (Ben-Amotz and Avron, 1972; Loeblich, 1972), and cell synchronization (Wegmann and Metzner, 1971).

Most algologists have tried to regulate the NaCl concentration of the culture media but have paid little attention to other parameters besides recognizing a minimal need and trying to satisfy this need in order to make their system work. Few definitive studies have been done concerning environmental regulation of growth of halophylic algae. High cell densities could be obtained but considerable time was required because of the slow growth rate. Usually one environmental factor or another was not considered, resulting in slow growth. The conditions that must be considered in order to regulate a population are both physical and chemical, and include the following: (1) culture media including chemical elements and their concentrations; (2) source and amount of carbon; (3) illumination; (4) temperature; (5) pH; and (6) salt concentration. Each of the above factors will be considered in the following experiments.

MATERIALS AND METHODS

A series of collections were made from the brine of the Great Salt Lake, Utah, U.S.A. After several unsuccessful attempts, unialgal cultures of an unidentified species of algae were obtained. According to H. C. Bold (Botany Dept., University of Texas, Austin, personal communication) and E. A. George (The Culture Centre of Algae and Protozoa, Cambridge, England, personal communication) the alga was an undescribed species of *Dunaliella*. These cultures were sustained in a medium containing major elements as described by Dyer and Gafford (1961) and minor elements in agreement with the work of Arnon (1938), plus 21% (w/v) NaCl. The *Dunaliella* cultures grew well once established, as did a number of halophylic bacteria. The medium was changed so that it was composed of the salts at the concentrations listed in Table I, resulting in better algal growth and a considerable reduction in the number of bacteria.

The next step in the isolation consisted of obtaining an axenic culture. This was accomplished by using the method of Wiedeman, Walne, and Trainor (1964) which consisted of washing the cells a number of times with sterile culture media containing a dilute detergent, spraying a dilute sample of algae on agar plates, and then picking and testing colonies for contamination. This method was very reliable.

TABLE I

Culture media

Salt	Concentration	Salt	Concentration
NaCl	175.329 g/l	$MnCl_2 \cdot 4H_2O$	1.81 mg/l
$Ca(NO_3)_2 \cdot 4H_2O$	0.662 g/l	$ZnCl_2$	0.11 mg/l
KH_2PO_4	0.313 g/l	$CuCl_2 \cdot 2H_2O$	0.050 mg/l
$MgSO_4$	0.277 g/l	$Na_2MoO_4 \cdot 2H_2O$	0.025 mg/l
Fe-EDTA	5.00 mg/l	$CoNO_3 \cdot 6H_2O$	0.050 mg/l
H_3BO_4	2.86 mg/l		

Stock cultures were kept on 1.5% plain agar (Bacto-Agar, Difco) which contained mineral elements as indicated in Table I and 17.5% (w/v) NaCl. Liquid cultures were grown in the same medium less agar. There were no vitamin supplements added to the medium and none are required, thus this species is a complete autotroph. Algal colonies were difficult to start on solid media, probably due to fragility of the cells because members of this genus do not have a cell wall. The cells cultured quite easily in liquid providing they were not exposed to harsh conditions during the transfer.

The alga cultures obtained were grown in mineral culture media aseptically and under a variety of conditions to determine their optimal growth response. Culture tubes 25 × 260 mm were used to raise the alga. The tubes were suspended from a $\frac{3}{8}''$ plexiglass cover into a water bath. The water bath was thermo-regulated between 5° and 40° C ± 0.5° C depending on the desired condition by balancing the flow of cold tap water (flowing through a copper coil) with a heating element connected to a thermostat. Lower temperatures were obtained by using a recycling refrigeration unit.

Compressed air and CO_2 were added to the culture tubes at a rate of 2.18 ml/min/ml culture medium at concentrations from 0.0–4.4% CO_2 (v/v) at 8 psi. The dry mixture of gas was filtered first through activated charcoal (40 mesh), second through sterile cotton, before it was saturated with water, and finally delivered to the culture through a 0.034″ diameter polyethylene tubing which was inserted into the culture through a plug. The light source at the lower intensities consisted of two 40-watt cool white fluorescent lamps; at higher intensities the source included four high intensity white fluorescent lamps. Light intensities were measured with a GE Light Meter or a Weston Illumination Meter Model 75. The suspension were maintained on a 20–4 hour light-dark period (to maintain high growth rates). Sodium chloride in the media was varied between 0.0 and 35% (w/v) as required.

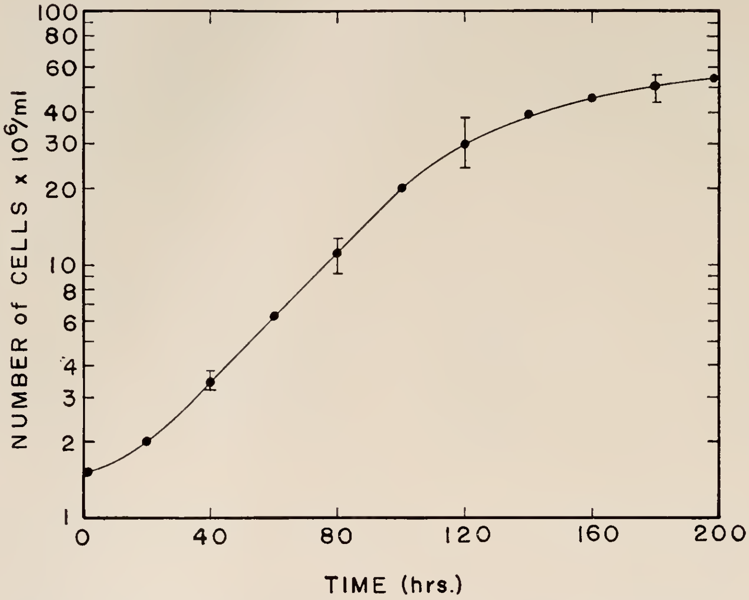

FIGURE 1. Growth curve for *Dunaliella* sp.; temperature 30° C, 21.0% NaCl, 2% CO_2, initial pH 5.5, light intensity 5.35 Klux (1 Klux = 93.4 ft-c), and a 20–4 hr light-dark cycle. Each point represents the mean of at least five determinations and the vertical bars represent ± one standard deviation.

The pH of the culture media was adjusted to 5.5 with 0.1 M NaOH and was then measured during the entire growth cycle (the pH could not be maintained more neutral because of the high CO_2 levels). A Beckman Expandomatic pH meter was used for pH determination. No Na^+ correction was needed in the range of pH's measured (Beckman Instruments, Inc., 1950). A specific buffer was not used in the media.

Cell populations were measured at various time intervals using a model "F" Coulter Counter with a 100 μ aperture (Coulter Electronics, Inc, 1967). The data were then plotted and from the resultant growth curve (Fig. 1) the specific growth rate (k) was calculated from which the doubling time (t_2) was determined (Myers, 1962). Next the t_2's were plotted *versus* the magnitude of the variable being investigated. The Coulter Counter worked very well in determining cell populations, but it was time consuming; consequently, a Beckman Model "B" spectrophotometer was calibrated at 750 nm using known cell dilutions. A standard curve was plotted and then the Model "B" was employed to make turbidometric measurements of the algal populations.

Carbon dioxide concentrations present in the ambient air were measured with a Beckman Model 215 A IR Gas Analyzer using standard CO_2 mixtures for comparison. A zero CO_2 concentration was obtained by passing the ambient air through three 2-liter flasks containing 1200 ml of 20% (w/v) KOH. Concentrations of CO_2 above ambient were obtained by mixing compressed CO_2 at a given temperature, pressure and flow rate with ambient air at the same temperature, pressure and a flow rate calculated to give the desired final concentration.

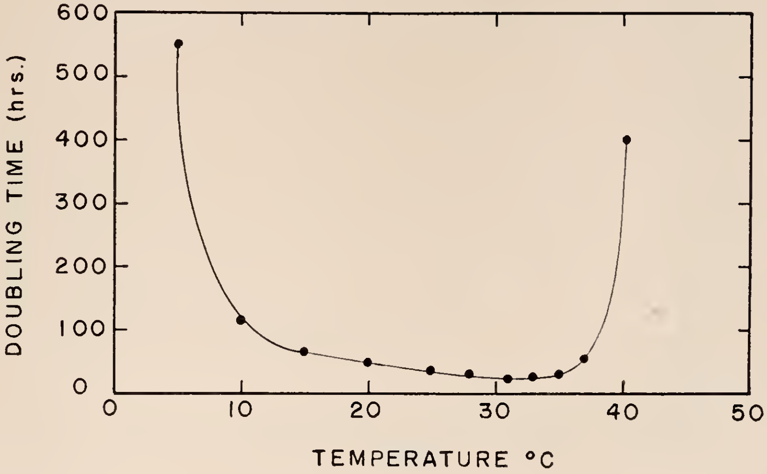

FIGURE 2. The effect of temperature on the doubling time (t_2). Growth conditions were: 21.0% (w/v) NaCl, 2% CO_2, initial pH 5.5, light intensity 5.35 Klux, and a 20–4 hr light-dark cycle. Each point represents the mean of at least five determinations.

For experiments on the sodium requirements of the algae, the cells were grown in 19.2% (w/v) or 28.0% (w/v) NaCl, and then centrifuged and resuspended in a small volume of the growth media. The cell population was determined and an aliquot of media containing 1×10^6 cells was placed in complete culture media with altered K^+/Na^+ ratios varying from 10^3 to 10^{-3}. Environmental conditions were optimal for growth. The doubling times were determined and plotted against the log of the K^+/Na^+ ratio.

All of the experiments were begun by inoculating five culture tubes with a large enough volume of inoculum so that the initial concentration of cells was 1×10^6/ml. The growth of the cultures was monitored spectrophotometrically and the data recorded and plotted. The doubling times were determined from the growth curves and finally plotted. In some cases, standard deviations were calculated and are shown in the appropriate figures.

RESULTS

Optimum temperature for growth was determined for the cells in 19.2% (w/v) NaCl medium at pH 5.5, 2% CO_2, 5.35 Klux and over a temperature range from 5° C–40° C (Fig. 2). Cultures were inoculated with 1 × 10^6 cells/ml. Cell populations were measured, growth rates were calculated, at least five replications were used, and the averages of the t_2's were then plotted *versus* temperature. Maximum growth took place at 32° C with the $t_2 = 23.8$ hrs. At 5° C and 40° C, the t_2's were 550 hrs and 415 hrs, respectively. At the lower temperature the cells were normal and active, but had a greatly decelerated growth rate. At the higher temperature the cells became distorted and abnormally large, probably due to damage to cell enzymes, particularly those involved with the osmoregulation system.

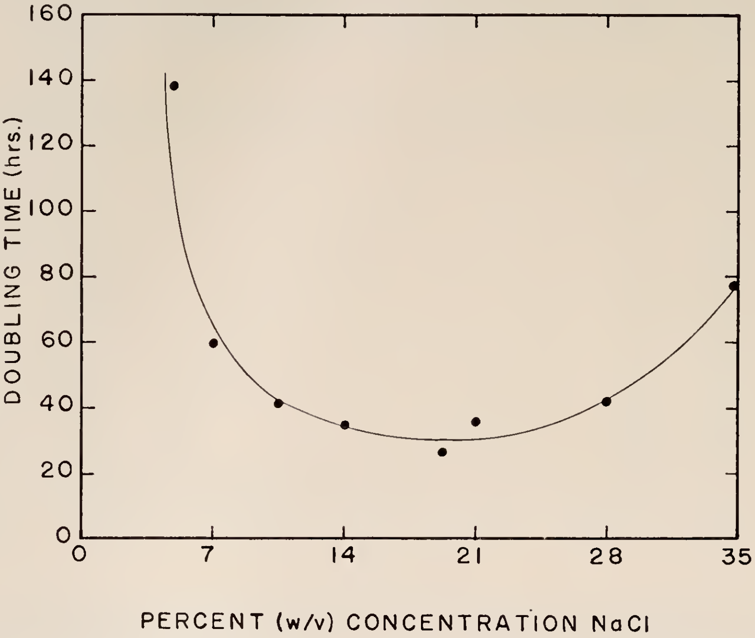

FIGURE 3. The effect of the NaCl concentration on the doubling time. Growth conditions were: 30° C, 2% CO_2 initial pH 5.5, 5.35 Klux, and a 20–4 hr light-dark cycle. Each point represents the mean of at least five determinations.

Cells were grown at 30° C, pH 5.5, 2% CO_2, 5.35 Klux light intensity and in culture medium ranging in concentration from 0.0 to 35% (w/v) NaCl in order to define the optimum NaCl concentration for growth (Fig. 3). Inoculation of cultures and the number of experiments were as previously described except when the NaCl concentration was changed. If the NaCl concentration was changed, it was accomplished by dilution with distilled water, or by addition of culture media with a higher NaCl concentration, and the change was not more than 3.5% at any one time. Changes were completed in a six-hour period, but the cells were not transferred to begin a new growth experiment for another four to six hours. When the cells were transferred it was noted that the lag time was short, comparable to those cultures not transferred, indicating no damage to the cells due to change in osmotic environment. Maximum growth occurred in 2.75 M or 19.2% (w/v) NaCl with a t_2 of 30 hrs. The t_2's were infinity for 3.15% (w/v) NaCl and 77 hrs for 35% NaCl. At NaCl concentration lower than 3.15% the cells did not grow, thus a death rate occurred rather than a growth rate, and this is not plotted in Figure 3.

Below 7% (w/v) NaCl the doubling time was very hard to determine because of "frothing" of the cultures. The cells clumped together and would not remain in suspension; consequently, there were very few measurements in this range and a high degree of variability.

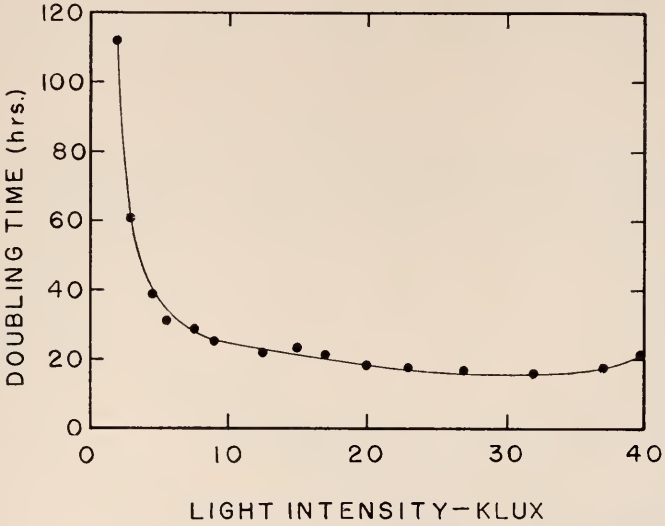

FIGURE 4. The effect of the light intensity on the doubling time. Growth conditions were: 30° C, 2% CO_2, initial pH 5.5, NaCl 19.2% (w/v), and a 20–4 hr light-dark cycle. Each point represents the mean of at least five determinations.

In order to measure the optimum light intensity the k was determined for the algae grown in 19.2% (w/v) NaCl culture medium at 30° C, pH 5.5, 2% CO_2, and over a range of light intensities from 2.12 Klux to 40.6 Klux (Fig. 4). The inoculation of cultures and the number of experiments were as described previously. Maximum growth took place between 25 and 35 Klux, and at this intensity $t_2 =$ 16 hrs. The doubling time approached infinity at a light intensity less than 1 Klux. A decrease in the growth rate also occurred at high light intensities (35–40 Klux), probably due to pigment bleaching or photo-oxidation.

The t_2 for the algae was also investigated at CO_2 concentrations varied from 0.0% to 4.4% (v/v) (Fig. 5), and the optimum concentration was determined. The light intensity was held constant at 26.8 Klux (technical difficulties caused the utilization of a slightly lower than optimum intensity), the temperature at 30° C, NaCl concentration at 19.2% (w/v) and the pH, inoculation, and number of replica-

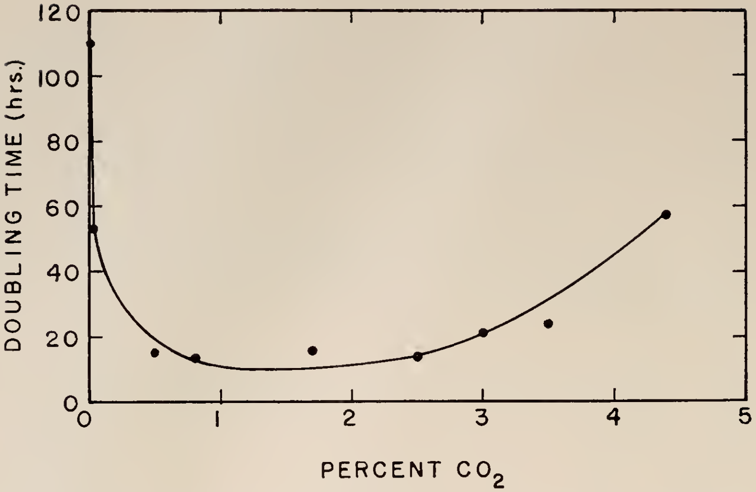

FIGURE 5. The effect of the CO_2 concentration on the doubling time. Growth conditions were: 30° C, intial pH 5.5, light intensity 26.8 Klux, NaCl 19.2% (w/v), and a 20–4 hr light-dark cycle. Each point represents the mean of at least five determinations.

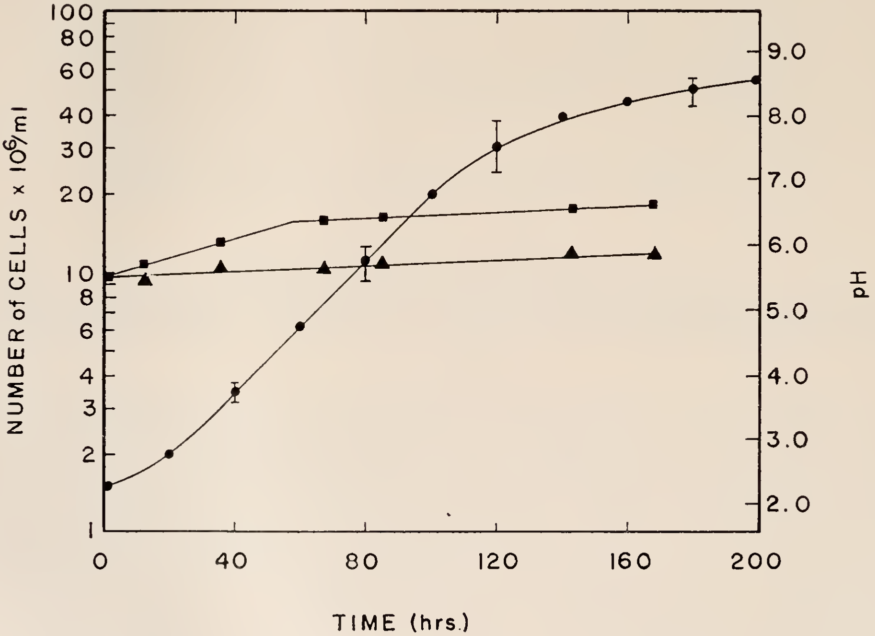

FIGURE 6. The effect of algal growth on the pH of the growth media, and the effect of the pH on the growth curve. Growth conditions were as follows: 30° C, 21.0% (w/v) NaCl, 2.0% CO_2, 5.35 Klux. Cells/ml (solid circles); pH of medium (solid squares). Each point represents the mean of at least five determinations, and the vertical bars represent ± one standard deviation; aerated uninoculated media (solid triangles).

tions were maintained as previously noted. Under these conditions maximum growth took place between 1 and 2% CO_2, with $t_2 = 10$ hrs.

The pH of the growing culture was determined at the same time as the growth rates were measured to see if any change in the pH of the culture occurred and to determine if the change in pH caused a change in the growth rate. The results of the experiment are plotted in Figure 6. The number of cells/ml is plotted on the left-hand vertical axis and the pH on the right-hand vertical axis, and both are plotted against time. The uninoculated aerated culture medium showed a small pH change (Fig. 6). The culture medium inoculated with 1.5×10^6 cells/ml increased in pH at the rate of 1.56×10^{-2} pH units/hr up to 60 hours and a pH of 6.4. After 60 hours, the pH rose very slowly 2.2×10^{-3} pH units/hr up to 170 hours and a pH of 6.5. Figure 6 shows the growth curve and the pH curve plotted against time. One can see that the pH over the range 5.5–6.5 has no effect on the log phase of the growth curve. The same figure shows that the pH is altered rather markedly by the presence of the algae.

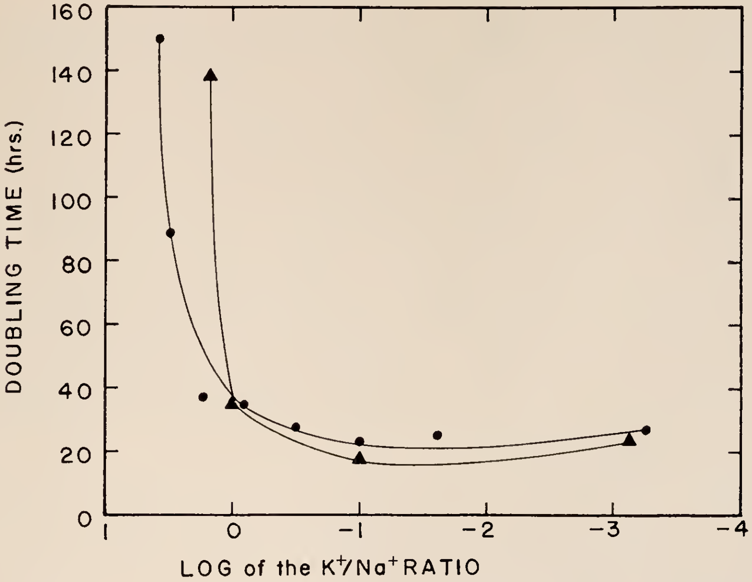

FIGURE 7. The effect of the K^+/Na^+ ratio at 19.2% (solid triangles) and 28.0% (solid circles) on the doubling time. Each point represents the mean of at least five determinations.

Figure 7 shows the effect of the K^+/Na^+ ratio on the doubling time. The optimum t_2 occurred between a K^+/Na^+ ratio of 0.1 and 0.001 and decreased at both lower and higher ratios. At the K^+/Na^+ ratio of 1.0 the k decreased very rapidly The same phenomenon occurred in both 19.2% and 28.0% salt concentrations.

DISCUSSION

The halophylic green algae are remarkably similar to the marine and freshwater algae in their requirements and growth response, except for their high requirement for an osmotically active substance, usually NaCl.

Innumerable formulas exist for media, variously selected by different laboratories (Society of Protozoologists, 1958). In the present study, most of the elements listed by Nicholas (1963) were used in suitable concentrations and the NaCl concentration was adjusted to that at which near optimal growth took place. Other authors (Gibor, 1956; Provasoli, McLaughlin and Droop, 1957; Yamada and Okamoto, 1961; Johnson *et al.* 1968) have used similar techniques.

All halophylic algae investigated have been grown at temperatures between 14° C and 30° C (Gibor, 1956; McLachlan, 1960; Yamada and Okamoto, 1961; Johnson *et al.,* 1968). From the above data and the data collected in the present study, it appears that the halophytes are mesothermal species. The growth rates of the undescribed *Dunaliella* were decreased at temperatures above and below 32° C, but 32° C was optimum for this species.

Light intensities that have been used to culture algal halophytes range from 1–22 Klux (Gibor, 1956; McLachlan, 1960; Yamara and Okamoto, 1961; Johnson *et al.,* 1968; Wegmann, 1971; Loeblich, 1972). At all but the highest intensities growth may actually be limited by low light intensity, but a low CO_2 concentration would be limiting at a higher intensity. In the present study, the t_2 was very low at light intensities of 1–5 Klux, optimum light intensity being about 25–35 Klux, but these measurements were made at high CO_2 concentrations. At the higher light intensity and higher CO_2 concentration, the t_2 was 16 hrs, which was a shorter doubling time than reported for any other halophlic algae.

The pH of the Great Salt Lake was reported between 7.4–8.4 (Flowers and Evans, 1966), depending on the location and the ions present. The marine environment also falls within this same range (Sillen, 1967). The pH of all reported growth experiments with halophylic species of *Chlamydomonas* and *Dunaliella* have been carried out between pH 7 and 9 (Gibor, 1956; Provasoli, McLaughlin and Droop, 1957; McLachlan, 1960; Yamada and Okamoto, 1961; Johnson *et al.,* 1968; Wegmann, 1971; Loeblich, 1972).

The species of *Dunaliella* used in this study has a wide range of pH tolerance. The growth conditions suggest the cells are not affected by broad changes in pH (5.5–6.5). Other experiments (to be reported elsewhere) show that there is very little change in the photosynthetic or respiratory rate from 4.5–8.5 pH units.

Provasoli, McLaughlin and Droop (1957) suggest 4% CO_2 enrichment for growing marine algae. For many species of algae carbon as glucose or acetate may be provided rather than CO_2 (Myers, 1962). However, some organisms are obligate photo-autotrophs and cannot utilize organic carbon from the culture medium.

All of the halophylic species of green algae investigated seem to be completely autotrophic (Gibor, 1956; McLachlan, 1960; Yamada and Okamoto, 1961; Johnson *et al.,* 1968; Wegmann, 1971; Loeblich, 1972). In all of the above cases, if CO_2 enrichment had been used coupled with higher light intensities (previous discussion), the t_2 would probably have decreased, indicating a shorter doubling time. The species reported here responded very favorably to increased CO_2 concentration. A high degree of stimulation occurred with a relatively small change in CO_2 concentration. An optimum growth rate occurred at 1–2% CO_2 at a given flow rate and pressure, but these cells were grown at elevated light intensities. A lowered pH at high CO_2 levels could have caused the increase in doubling time, but this information is not available at the present time.

Salt concentrations from 0–35% NaCl have been used to culture the brine algae (McLachlan, 1960; Yamada and Okamoto, 1961; Johnson *et al.*, 1968; Wegmann, 1971; Wegmann and Metzner, 1971; Loeblich, 1972). Optimum growth occurred at 19.2% NaCl for the unidentified *Dunaliella* reported in this paper, thus it is physiologically separate from other members of the genus. The t_2 was maximum at this concentration but there was a wide range of tolerance. The greatest effect of NaCl concentration occurs at the low salinity range, and the t_2 increases rapidly below 7.0% NaCl. The osmoregulation system may be overloaded at the low concentration of NaCl; or there may be a leakage of the plasma membrane or an increase in secretion of metabolic constituents into the media. There is some evidence of the latter effect shown by an apparent increase in viscosity of the growth medium.

Cells growing in environments containing a high concentration of Na^+ could have a definite Na^+ requirement. McLachlan (1960) showed a Na^+ requirement for *D. tertiolecta*, but generally it is very difficult to prove a definite requirement of a metal ion for a given organism (Arnon, 1938), especially for such a common metal ion as Na^+. This experiment (Fig. 7) does not show an absolute Na^+ requirement, but it shows a definite dependence on the amount of Na^+ in the medium and suggests that the ion cannot be substituted for, at least by K^+ at the concentrations used in these experiments. There is a possibility that another osmotic agent could be used to substitute for the Na^+ or both the Na^+ and the Cl^-. A compound like glycerol, carbowax (polyethylene glycol), sugar, or sugar alcohol, or even another alkali or alkali earth salt could be used. These experiments have not been carried out to date because of two problems: one being toxicity of the substitute compound; and the other being low solubility.

With cells grown in a 2.75 M salt media, the optimum t_2 occurred at a K^+/Na^+ ratio of 0.01, but at a 1/1 K^+/Na^+ ratio the t_2 decreased very rapidly. As the K^+ concentration increased and the Na^+ concentration decreased, the specific growth rate decreased. This suggested that at least the K^+ to Na^+ ratio was very important; and once a certain level is reached, the cells do not function properly. Possibly the internal K^+ concentration was at such a high level it inhibited certain enzymatic reactions by substituting for Na^+ at the active site. Sorger, Ford and Evans (1965) showed a definite requirement of some enzymes of mesophytes for Na^+ or K^+. They reported that some substitution of ions could occur, but the activity was reduced. Another possibility could be that K^+ was actively absorbed until a much higher internal K^+ concentration occurred; and if this happened, the high ionic strength of the K^+ could precipitate some enzymes, or change their charge distribution and cause them to be inactive or to have a lower activity. The cause of the decrease in the specific growth rate is not known, but the experiment showed that the cells would not grow in a high K^+ medium; however, they would grow in a high Na^+ medium, suggesting a Na^+ requirement. At the same time, there was a slight increase in the t_2 at low K^+ concentrations suggesting a K^+ requirement. The cells in these experiments were not killed by osmotic shock because changes in osmodicum occurred over an eight to twelve-hour period.

The same experiment was carried out at a 4.0 M concentration, and the results were very similar. The decrease in growth rate at a higher salt concentration was expected and occurred in other experiments. This decrease could have been due to a higher internal NaCl concentration, an osmotic response, and/or a reduction in enzyme activity (Johnson *et al.,* 1968), or it could have been due to a lower P/R ratio, resulting from a greater energy expenditure for osmoregulation (McLusky, 1969.) The optimum doubling occurred at the same K^+/Na^+ ratio and a very steep decline occurred at a K^+/Na^+ ratio of 1.0. The doubling time increased to infinity at 0.1 M and beyond that point the specific growth constants were negative (death rates) and are not shown in Figure 7, but the magnitude of decrease was not as great as in the 2.75 M medium. One possibility to be considered is that even though the K^+/Na^+ ratio was the same, there were more Na^+ ions present at the higher molarity than there were in the 2.75 M media thus modifying the deleterious effect.

The optimum conditions for the growth of this species of *Dunaliella* as determined in these experiments are as follows: NaCl 19.2%, CO_2 1–2% delivered at a rate of 2.18 ml/min/ml culture solution, temperature 32.0° C, light intensity 25–35 Klux, and a pH range from 5.8–6.5. Under these conditions the doubling time (t_2) was 10 hours. The optimum conditions described here ignore any interaction between factors tested.

The specific growth rate and the doubling time for optimal growth can be compared directly with freshwater, marine or other brine algae. Specific growth rates and t_2's reported were not greatly different from those reported for other species of algae grown under optimum conditions (McLachlan, 1960; Yamada and Okamoto, 1961; Talling, 1962; Wilson and Lords, 1965; Fogg, 1965; and Loeblich, 1972).

Of all the factors tested the one most limiting was CO_2 concentration (Fig. 5). A very slight increase in CO_2 concentration over ambient caused a very marked change in the t_2 (Fig. 5). This dramatic increase in growth rate did not occur for any of the other variables tested, at least not in the "normal" physiological or ecological range, but at the limits drastic changes in doubling time occurred.

This species is not unusual because it grows and divides at a given rate, a rate at least equal to that of many freshwater and marine forms; but that it can grow and divide at this rate in a saturated NaCl medium, and that growth does not occur at all in media containing less than 3.15% NaCl.

One of the postulated reasons for a maximum growth rate at a high salt concentration for the algae is that the internal salt concentration is lower than that of the medium (Yamada and Okamoto, 1961; Johnson *et al.*, 1968). If this is the case, another active osmotic agent must be present, and evidence has been presented to show that glycerol may be this agent (Craigie and McLachlan, 1964; Jokela, 1969; Wegmann, 1971). Next, one should ask why the cells do not grow well at salt concentrations higher or lower than optimum. This point remains to be investigated; but possibly the osmoregulation system is inhibited at higher and lower salt concentrations due to a breakdown of the control caused by a reduced energy supply (inhibited respiration or photosynthesis), decreased glycerol production or alterations of the Na^+/K^+ pumping or regulatory system which may lead to the rupturing of the cells, or reduced growth rates. There may be other factors involved, but they are not known at the present time.

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SUMMARY

A halophylic species of *Dunaliella* was isolated from the Great Salt Lake, Utah, and established in axenic culture. A balanced culture media was developed containing major and minor elements as well as a sufficient concentration of an osmotic agent. The effects of various environmental factors on the growth of this species of algae were investigated and optimum growing conditions were delineated. Optimum conditions for growth of this species are as follows: (1) temperature 32° C; (2) NaCl 19.2% (w/v); (3) CO_2 1–2% at a rate of 2.2 ml/min/ml of culture media; (4) light intensity 25–35 Klux; and (5) pH 5.8–6.5. The K^+/Na^+ ratio should not be more than one, and better growth took place when this ratio was less than 0.1. The specific growth constant for this halophyte under the above conditions was 0.069 hrs^{-1}, which is equal to a doubling time of 10.0 hours.

LITERATURE CITED

Arnon, D. I., 1938. Microelements in culture-solution experiments with higher plants. *Amer. J. Bot.*, **25**: 322–325.

Baas-Becking, L. G. M., 1931. Salt effects on swarmers of *Dunaliella viridis* Teod. *J. Gen. Physiol.*, **14**: 765–779.

Beckman Instruments, Inc., 1950. Instructions for the use of Beckman glass electrodes made with general purpose glass. *Beckman Bull.*, **255-A**: 2.

Ben-Amotz, A., and M. Avron, 1972. Photosynthetic activities of the halophilic alga *Dunaliella parva*. *Plant Physiol.*, **49**: 240–243.

Butcher, R. W., 1959. An introductory account of the smaller algae of British coastal waters. *Fish. Invest. Minist. Agric. Fish. Food (Gr. Brit.) Series IV*, **Part 1**: 21–24.

Craigie, J. S., and J. McLachlan, 1964. Glycerol as a photosynthetic product in *Dunaliella tertioleicta* Butcher. *Can. J. Bot.* **42**: 777–778.

Coulter Electronics, Inc., 1967. *Coulter Counter Instructions and Service Manual for the Model "F"*. Coulter Electronics, Inc., Hialeah, Florida, 125 pp.

Dyer, D. L., and R. D. Gafford, 1961. Some characteristics of a thermophilic blue-green alga. *Science*, **134**: 616–617.

Flowers, S., and F. R. Evans, 1966. The flora and fauna of the Great Salt Lake region, Utah. Pages 367–393 in Hugo Boyko, Ed., *Salinity and Aridity; New Approaches to Old Problems*. W. Junk, The Hague.

Fogg, G. E., 1965. *Algal Cultures and Phytoplankton Ecology*. University of Wisconsin Press, Madison, 126 pp.

Gibor, A., 1956. The culture of brine algae. *Biol. Bull.* **111**: 223–229.

Johnson, M. K., E. J. Johnson, R. D. MacElroy, H. L. Speer and B. S. Bruff, 1968. Effects of salts on the halophilic alga *Dunaliella viridis*. *J. Bacteriol.*, **95**: 1461–1468.

Jokela, A. C. T., 1969. Outer membrane of *Dunaliella tertiolecta*: isolation and properties. *Dissertation, University of California, San Diego*, 123 pp.

Kirkpatrick, R., 1934. Life of the Great Salt Lake with special reference to the algae. *Unpublished Master's thesis, University of Utah*, 30 pp.

Latorella, A. H., 1971. Growth and salinity adaptation in the phytoflagellate, *Dunaliella tertiolecta*. *Dissertation, University of Maine*, 106 pp.

Loeblich, L. A., 1972. Studies on the brine flagellate *Dunaliella salina*. *Dissertation, University of California, San Diego*, 157 pp.

Marrè, E., and O. Servettaz, 1959. Sul meccanismo di adattamento a condizioni osmotiche estreme in *Dunaliella salina*. II. Rapporto fra concentrazioni del mezzo esterno e composizione del succo cellulare. *Atti Accad. Naz. Lincei Rend. Cl. Sci. Fis. Mat. Natur.*, **26**: 272–278.

McLachlan, J., 1960. The culture of *Dunaliella tertiolecta* Butcher—a euryhaline organism. *Can. J. Microbiol.*, **6**: 367–379.

McLusky, D. S., 1969. The oxygen consumption of *Corophium volutator* in relation to salinity. *Comp. Biochem. Physiol.*, **29**: 743–753.

Myers, J., 1962. Laboratory cultures. Pages 603–615 in R. A. Lewin, Ed., *Physiology and Biochemistry of Algae.* Academic Press, New York.

Nicholas, D. J. D., 1963. Inorganic nutrient nutrition of microorganisms. Pages 363–447 in F. C. Steward, Ed., *Plant Physiology; Vol. III; Inorganic Nutrition of Plants.* Academic Press, New York.

Okamoto, H., and Y. Suzuki, 1964. Intracellular concentration of ions in a halophilic strain of *Chlamydomonas.* I. Concentration of Na, K, and Cl in the cell. *Z. Allg. Mikrobiol.*, **4**: 350–357.

Provasoli, L., J. A. McLaughlin and M. R. Droop, 1957. The development of artificial media for marine algae. *Archiv. Mikrobiol.*, **25**: 392 428.

Sillen, L. G., 1967. The ocean as a chemical system. *Science*, **156**: 1189–1197.

Smith, G. M., 1950. *The Fresh-Water Algae of the United States.* [2nd ed.] McGraw-Hill, New York, 719 pp.

Society of Protozoologists, 1958. A catalogue of laboratory strains of free-living and parasitic protozoa. *J. Protozool.*, **5**: 1–38.

Sorger, G. J., R. E. Ford and H. J. Evans, 1965. Effects of univalent cations on the immunoelectrophoretic behavior of pyruvic kinase. *Proc. Nat. Acad. Sci.*, **54**: 1614–1621.

Talling, J. F., 1962. Freshwater algae. Pages 743–757 in R. A. Lewin, Ed., *Physiology and Biochemistry of Algae.* Academic Press, New York.

Wegmann, K., 1971. Osmotic regulation of photosynthetic glycerol production in *Dunaliella.* *Biochim. Biophys. Acta*, **234**: 317–323.

Wegmann, K., and H. Metzner, 1971. Synchronization of *Dunaliella* cultures. *Arch. Mikrobiol.* **78**: 360–367.

Wiedeman, V. E., P. L. Walne and F. R. Trainor, 1964. A new technique for obtaining axenic culture of algae. *Can. J. Bot.*, **42**: 958-959.

Wilson, D. G., and J. L. Lords, 1965. Growth characteristics of *Microcystis aeruginosa* cultured at elevated temperatures. *Proc. Utah Acad. Sci. Arts Lett.*, **42**: 159–160.

Yamada, H., and H. Okamoto, 1961. Isolation and pure culture of a halophilic strain of *Chlamydomonas.* *Z. Allg. Mikrobiol.*, **1**: 245–250.

Yamamoto, M., 1967. Osmotic regulation in a halophilic strain of *Chlamydomonas.* II. Effects of sulfhydryl reagents. *Z. Allg. Mikrobiol.*, **7**: 267–277.

Yamamoto, M., and H. Okamoto, 1967. Osmotic regulation in a halophilic *Chlamydomonas* cell. I. General feature of the response to the change in osmotic pressure. *Z. Allg. Mikrobiol.*, **7**: 143-150.

Yamamoto, M., and H. Okamoto, 1968. Osomotic regulation in a halophilic *Chlamydomonas* cell. III. Mg^{++} — and Ca^{++} — dependent adenosine-triphosphatase in microsome fraction. *Z. Allg. Mikrobiol.*, **8**: 71–84.

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ELECTRICAL ACTIVITY AND BEHAVIOR IN THE SOLITARY HYDROID *CORYMORPHA PALMA*. I. SPONTANEOUS ACTIVITY IN WHOLE ANIMALS AND IN ISOLATED PARTS

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Even before the advent of electrical recording, cnidarians, with their simple nervous systems and apparently simple behavior, attracted many investigators interested in analyzing the physiological basis of behavior. Following Josephson's (1961b) demonstration that it was possible to record large, spontaneous electrical potentials from hydroids, a number of investigators turned their attention to analysis of the relation between electrical activity and behavior in these organisms with the result that this relationship has now been investigated in *Tubularia* (Josephson and Mackie, 1965; Josephson and Uhrich, 1969). *Hydra* (Passano and McCullough, 1962, 1963, 1964, 1965; Rushforth 1971; Rushforth and Burke, 1971); *Cordylophora* (Josephson 1961a, 1961b; Mackie, 1968); and *Obelia* (Morin and Cooke, 1971a, 1971b). An association between behavior and at least one type of spontaneous electrical activity was observed in all of these hydroids except *Cordylophora*.

From Parker's (1919) description it appeared that *Corymorpha* could exhibit fairly complex behavior as compared with other hydroids. This description and the conclusion by Parmentier and Case (1973) that there was, at best, an indirect relation between activity and behavior in the closely related hydroid *Tubularia* led to my investigation of the relation between electrical activity and behavior in *Corymorpha palma* Torrey. *Corymorpha* is especially favorable for such an investigation because of its large size and its diminutive perisarc, which usually covers less than a fifth of the stalk.

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Materials and Methods

Specimens of *Corymorpha palma* Torrey (Fig. 1) were collected using SCUBA at depths of up to fifteen feet in Newport Bay, California. They were maintained in aquaria with either open or recirculating sea water systems at temperatures of 12°–23° C and an illumination cycle of 16 hours light and 8 hours of darkness. *Artemia* nauplii were added to each aquarium every day or two.

Gross morphology of *Corymorpha* was investigated by dissection, while for cellular details tissue was fixed in picroformol, embedded in paraffin, sectioned, and stained with hematoxylin and triosin. Oxidized methylene blue staining for nerves was done on fresh tissue. Individual cell types were examined following maceration of fresh tissue in Bela Haller's macerating fluid (Gatenby and Painter, 1937).

For electrical recording from a whole animal, *Corymorpha* was placed on sand in the bottom of a culture dish (80 × 100 mm) full of sea water adjusted to 20 ± 1° C by immersion in a water bath. This temperature was maintained during

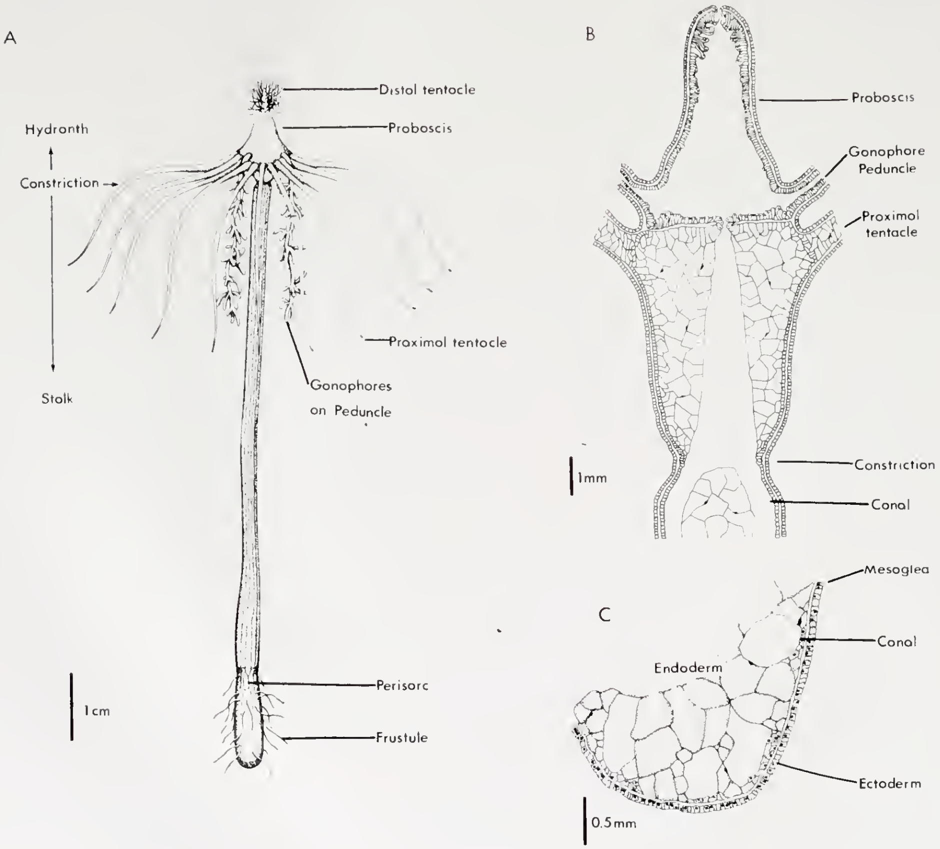
A
Distal tentacle
Hydranth
Proboscis
Constriction
Proximal tentacle
Stalk
Gonophores on Peduncle
Perisarc
Frustule
1 cm
B
Proboscis
Gonophore Peduncle
Proximal tentacle
1mm
Constriction
Canal
C
Mesoglea
Canal
Endoderm
Ectoderm
0.5mm

FIGURE 1. *Corymorpha palma* Torrey; (A.) whole animal showing various external features, (B.) longitudinal section of the hydranth, (C.) a partial cross section of the stalk.

recording by a recirculating water-bath or by working in a temperature-controlled room. Sand, which is held in place by the frustules, normally surrounds the bases of the animals, allowing them to be moved without too much disturbance and aiding them in regaining their normal upright stance. A 10–30 minute recovery period was allowed following the transfer of an animal to the culture dish. During this period the animal reassumed the upright posture normally seen in nature.

Electrical recording was done using suction electrodes made by drawing out flexible "Tygon" tubing (i.d. 0.625 mm) to a tip orifice in the range of 50–200 μm, as appropriate to record from the particular part of the animal which was under study. The indifferent electrode was a platinum-iridium wire in the bath. Electrical activity was monitored on a Tektronix 502A oscilloscope and recorded on a Beckman RB Dynograph after amplification with Grass P511 AC preamplifiers.

Excision experiments were performed on animals standing on sand in a culture dish. The normal pattern of electrical activity was first established and then one or more excisions were performed. *Corymorpha* can readily be sectioned at the neck and at the base of the proximal tentacles, making it possible to record from the following parts in various combinations: isolated proximal tentacles, isolated hydranth, and isolated stalk. Recordings were also made from excised gonophores and excised distal tentacles, but these were removed before recording was begun.

For studies on the effects of temperature on spontaneous electrical activity individual *Corymorpha* or their excised parts were placed in sea water on a sand bottom in an aluminum or glass container in a waterbath. Recordings were made from three intact animals, from three excised hydranths, and from six excised proximal tentacles from three different animals. Electrodes were placed in a variety of positions. The temperature of the waterbath was adjusted by adding ice or water of various temperatures and monitored by a themistor probe, placed in the sea water next to the animal, with the output recording on one channel of the Dynograph. Temperature changes were made at several different rates.

RESULTS

The structure of Corymorpha

My morphological and histological investigations have generally confirmed the previous findings of Torrey (1902, 1904a, 1904b, 1905, 1907, 1910a, 1910b, 1910c) and Parker (1917). However, the results will be briefly summarized here since they are necessary for an adequate understanding of the experimental work.

Figure 1 shows some of the more significant anatomical features of *Corymorpha palma*. An undisturbed animal in its natural environment may reach a height of over 30 cm. Only the bottom fifth or less of the stalk is covered with perisarc. Frustules, or holdfasts, which anchor the stalk in the sand, grow out at the sides of each endodermal canal in the area covered with perisarc. As shown in Figure 1C, the axis of the stalk is filled with large endodermal cells, and the gastrovascular cavity has been reduced to canals which are spaced around the periphery of the stalk. From the exterior of the animal these canals are apparent as longitudinal white streaks which frequently anastomose, especially toward their proximal ends. The ectoderm of the stalk is made up of typical epitheliomuscular cells (Figs. 2, 3) with the muscle fibers running longitudinally next to the meso-

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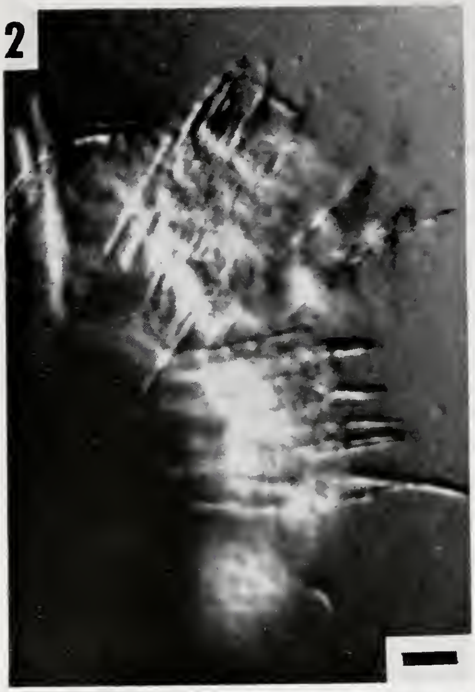

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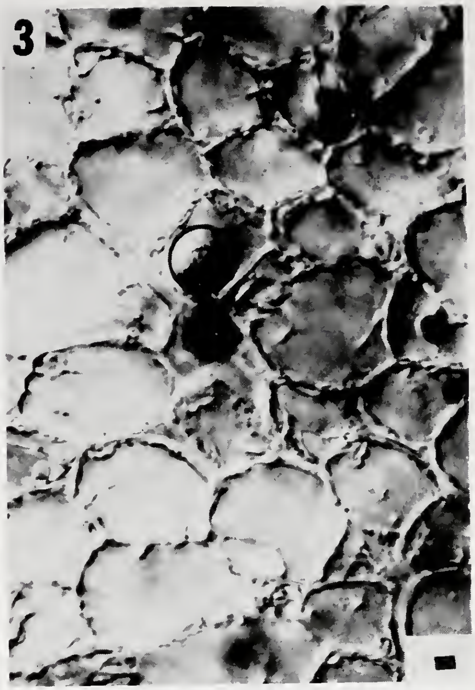

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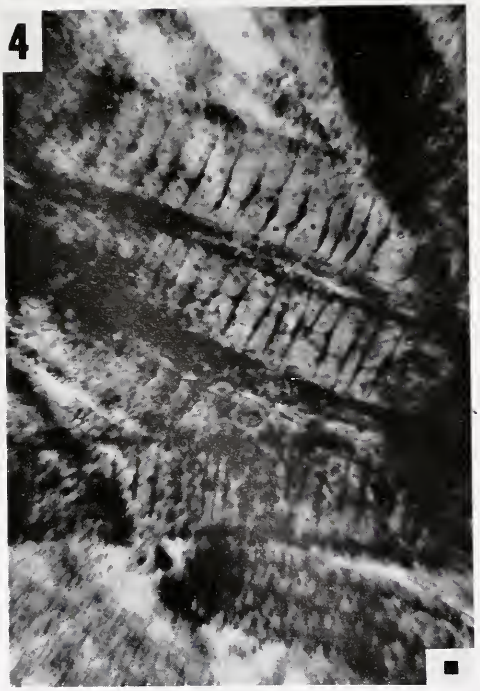

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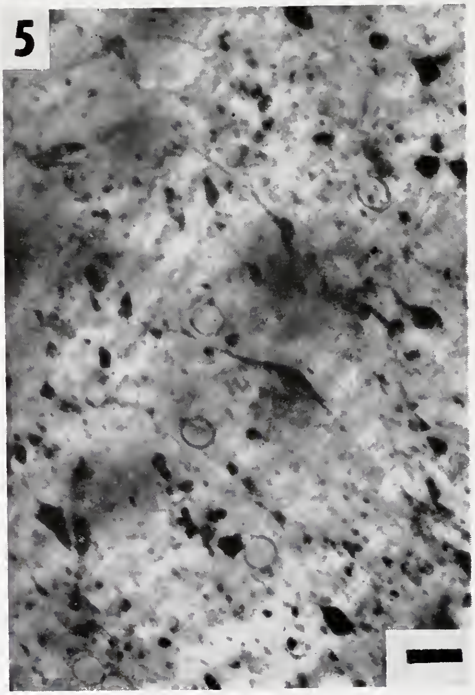

glea. Nematocysts are scattered among the epitheliomuscular cells and are more abundant on the naked distal part of the stalk.

The mesoglea forms a layer about 15 μm thick (Chapman and Pardy, 1972), beneath which lie the endodermal cells. These cells occur in a continuum of sizes, from the small cells lining the canals to the very large cells which extend into the axis of the stalk. Those portions of the endodermal cells lying against the mesoglea contain muscle fibers which constitute the circular musculature of the stalk. The axial endodermal cells, which are remarkable for their great size, have only a thin rind of cytoplasm around a huge vacuole. A marked constriction separates the stalk from the hydranth.

The proboscis of *Corymorpha* is a complex structure with a basic musculature similar to that of the stalk, there being an ectodermal longitudinal layer and an endodermal circular layer. Groups of endodermal cells at the bases of the proximal tentacles partially separate the hydranth cavity into two chambers (Fig. 1B), the lower of which connects with the canals of the stalk.

Twenty to thirty large proximal tentacles arise at equal intervals around the base of the proboscis. Each tentacle is organized in a manner basically similar to the stalk: thin layers of ectoderm and mesoglea surround the large endodermal cells which fill the axis (Fig. 4). Torrey (1904a) described both circular and longitudinal muscles in both proximal and distal tentacles, but stated that the circular muscles form a much weaker sheet except where each tentacle joins the body of the hydranth. Parker (1917) and G. Chapman (personal communication) were unable to find any evidence for circular muscles.

A variable number of hollow stems which bear gonophores arises just distal to the proximal tentacles. The term "gonophore peduncles" will be used for these structures since, according to Hyman (1940), it is debatable whether they represent true blastostyles.

A bare zone above the gonophore peduncles is terminated distally by forty to sixty small distal tentacles which cover the sides of the proboscis and surround the mouth located at its tip. These tentacles are solid and are organized in the same manner as the proximal tentacles.

No previous investigators have noted the presence of nerves in *Corymorpha*, although Parker (1917, 1919) assumed that a nerve net is present. Methylene blue staining revealed what appear to be typical coelenterate nerve cells (Fig. 5) lying in the ectoderm above the mesoglea. However, only isolated cells stained; hence there was not clear evidence for a well-developed net or for concentrated nerve tracts.

FIGURE 2. Epitheliomuscular cells following macreation in Bela Haller's macerating fluid—microscope focused to show basal muscle fibers, Nomarski optics; Scale = 10 μm.

FIGURE 3. Stalk ectoderm—a pavement epithilium formed by the upper part of the epithliomuscular cells. Note nematocyst, Nomarski optics; scale = 10 μm.

FIGURE 4. Large endodermal cells in proximal tentacles; scale = 10 μm.

FIGURE 5. Methylene blue-stained nerve cells in the stalk of *Corymorpha*; scale = 10 μm.

Behavior of the whole animal

In nature undisturbed *Corymorpha* are usually found standing relatively upright with the hydranth facing downstream when there is a current. At certain times of year the water of Newport Bay contains many strands of mucus from the gastropod, *Bulla,* and the proximal tentacles of *Corymorpha* may be festooned with these strands and the material trapped by them. From time to time one or more proximal tentacles will sweep inward carrying a small organism to the mouth or there may be some apparently random distal tentacle activity. In a few instances animals have been discovered with the mouth widely open and with the walls of the proboscis contracted longitudinally while a bolus of undigested material was regurgitated.

Concert and bowing behavior in *Corymorpha,* discussed in considerable detail

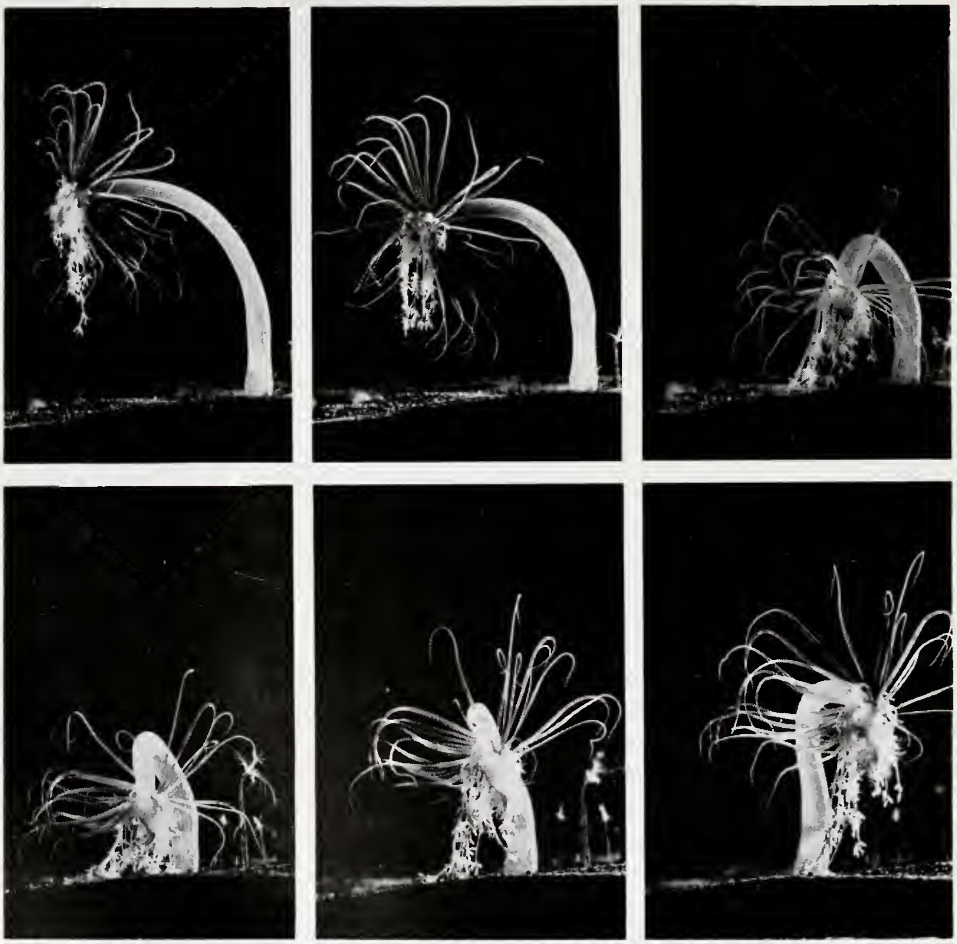

FIGURE 6. Bowing behavior in *Corymorpha.* Frames were taken at approximately 30-second interval and run from left to right, top to bottom.

by Parker (1917, 1919) appears to be limited to periods of quiet water; such periods are relatively short in the areas which I studied most thoroughly. Parker's observation on the inhibitory effect of a current was confirmed under field conditions by inversion of an aquarium over a portion of a bed of *Corymorpha,* all of which were standing erect in a current. Within a few minutes many of the animals under the aquarium began to show concert behavior and a variable degree of bowing, while their neighbors outside in the current continued to stand erect.

During concert behavior the proximal tentacles are simultaneously flexed inward around the proboscis from one to three times. Concurrently there is often a contraction of the stalk musculature. It appears that a majority of the polyps perform concert behavior sometime during any period of slack water. Some show this behavior quite regularly at intervals of 3–8 minutes, while others show no apparent rhythmicity.

Bowing behavior, if it occurs at all, normally occurs either simultaneously with stalk contraction or immediately thereafter. The bowing sequence shown in Figure 6 is atypical in that it did not start with stalk contraction and a concert. The degree to which an animal bows varies greatly from individual to individual. During the intervals when I was observing them, than 1% of the population bowed deeply enough to touch the distal tentacles to the substratum in the manner described by Parker (1917, 1919). Those animals which bowed deeply appeared to do so consistently.

The field and laboratory behavior which I have observed can be classified into individual acts and combinations of these acts such as:

1. Simultaneous outward and downward flaring of the distal tentacles, usually in response to some stimulus (distal opening response).
2. Longitudinal contraction of the proboscis.
3. Simultaneous inward flexion of all proximal tentacles.
4. Contraction of the longitudinal musculature of the stalk.
5. Bending of the stalk.
6. Peristaltic waves passing downward along the proboscis.
7. Inward movement of one or a few proximal tentacles.
8. Contraction of from one to many gonophore peduncles.
9. Bending of the proboscis to the side either spontaneously or in response to stimulation.
10. Apparently uncoordinated writhing or an oral flexion by some or all of the distal tentacles.
11. Opening and closing of the mouth.
12. Gaping of the mouth associated with regurgitation.

The list was still growing at the end of the study, so it should not be regarded as a complete enumeration of the acts of which *Corymorpha* is capable. Moreover, it is possible that some of the acts listed should be subdivided further. As it stands, however, the list summarizes the commonly observed behavior of *C. palma* and provides a way of discussing the individual acts which, together, encompass most of the behavior of the animal.

When possible the terminology established by Josephson and Mackie (1965) will be used whenever the behavior being described appears to be analogous to that

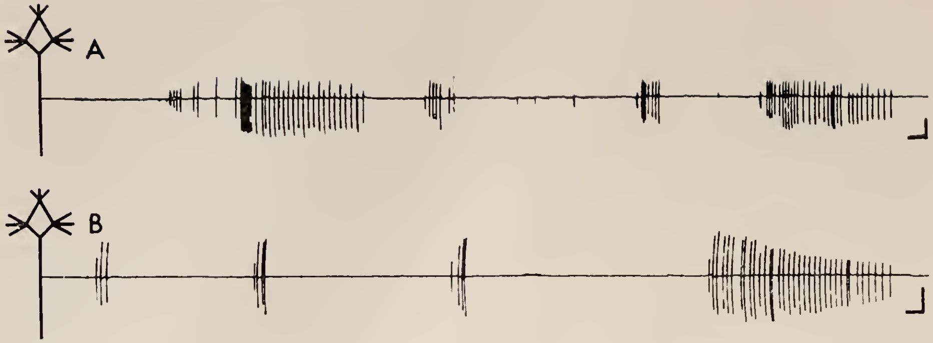

FIGURE 7. Stalk burst patterns in two *Corymorpha*. The approximate position of the recording electrodes is shown diagrammatically on the left; vertical scale equals 500 μV; horizontal equals 5 sec.

which they described in *Tubularia*. Following their usage, category (1) is termed a "distal opening response." Categories (2) and (3), which often occur in association with each other, constitute a "concert." A concert in conjunction with category (4) will be termed a "concert with stalk contraction." As previously noted, the degree of bowing varies greatly from individual to individual, so a "concert with stalk contraction" and a "concert with stalk contraction and bowing," category (5), must be regarded as the ends of a continuum with no clear separation between them. The other acts listed may occur independently and have not been given special names.

Electrical activity and its behavioral correlates in the whole animal

Following the usage set by Josephson and Mackie (1965) in *Tubularia,* the spontaneously active pulse systems in *Corymorpha* will be named according to the part of the animal in which the pulses appear to originate. Although "proboscis pulse" seems to be a more accurate term, the category "hydranth pulse" will be retained (a) because the pulses referred to under this title appear to be analogous in both *Tubularia* and *Corymorpha,* and (b) to avoid adding unnecessarily to the already extensive list of hydroid pulse types.

Pulse duration was measured from the initial departure from the recorded baseline to the final return to this baseline. Pulse amplitudes were measured from maximum negativity to maximum positivity. In all records negative potentials of the active electrode relative to the bath are displayed as downward deflections. The term "burst" is used in a rather broad sense to denote a temporally isolated series of two or more pulses. The number given in parentheses following each pulse category is a minimum estimate of the number of animals recorded from at that site.

Stalk pulses (*SP* > *50*). Stalk pulses usually occur in bursts which may vary widely in duration and pulse number (Table I). Stalk burst patterns are shown in Figure 7. The site of initiation of stalk pulses varies from time to time and from animal to animal. Waveforms vary greatly both within and between animals

TABLE I

Summary of Corymorpha *stalk burst characteristics: each line represents a separate animal*

No. bursts measured	Range of burst durations (sec)	Av. burst duration (sec)	Range of SPs/burst	Av. no. SPs	Av. pulse frequency (SPs/sec)
1	8	8	4	4	0.50
3	5.2– 13.6	8.3	11–16	13.0	1.57
6	2.0– 45.0	14.8	4–35	13.8	0.93
9	2.0– 69.0	18.6	4–44	14.5	0.78
7	2.0–106.0	22.2	2–52	16.4	0.74
5	10.0– 50.9	27.4	14–45	27.6	1.01
2	48.0– 61.0	54.5	25–27	26.0	0.48
2	52.8– 58.0	55.4	33–43	38.0	0.69
4	51.0– 76.0	59.3	33–64	46.8	0.79
1	152	152	55	55	0.36
Av. 4	33.3– 64.0	42.1	18.5–38.5	25.5	0.79

at least when recording with suction electrodes. Characteristics of stalk pulses and other pulse types are summarized in Table II. The average conduction velocity based on a minimum of 18 measurements on each of 5 animals was 15.9 cm/sec (range of individual averages 14.9–18.2 cm/sec).

A contraction of the longitudinal muscles of the stalk almost always occurs simultaneously with a stalk burst; but one or two pulses sometimes occur without any apparent behavioral correlate. In general there is a clear association between the length of a burst and the strength of the associated contraction. Usually there is a concert associated with stalk bursts; but excision experiments clearly show that this is due to the triggering effect of the stalk activity.

Although pulses may be triggered by electrical stimulation at any point along the stalk, spontaneous bursts normally appear to originate in its lower half. To further examine sites of pulse initiation five stalks were cut into 3 approximately equal sections, each of which was found to be capable of generating spontaneous bursts. However, long bursts of 20 or more pulses were limited to the basal

TABLE II

Summary of characteristics of the pulse types produced by Corymorpha. *Average values were obtained by measuring 10 pulses from each of 5 animals. Distal tentacle pulses were measured on excised hydranths, all other types on intact animals*

Pulse type	Duration (msec) $\bar{X}$ S	Range of individual averages (msec)	Amplitude (mV) $\bar{X}$ S	Range of individual averages (mV)
Distal Tentacle	63 ± 12	52–80	8.14 ± 5.23	3.78–15.95
Hydranth	68 ± 18	39–84	1.32 ± 0.84	0.34– 2.67
Proximal Tentacle	89 ± 27	51–125	1.72 ± 1.77	0.61– 4.85
Gonophore Peduncle	75 ± 23	48–109	1.52 ± 0.58	0.74– 2.25
Stalk	61 ± 13	42–73	0.89 ± 0.35	0.49– 1.37

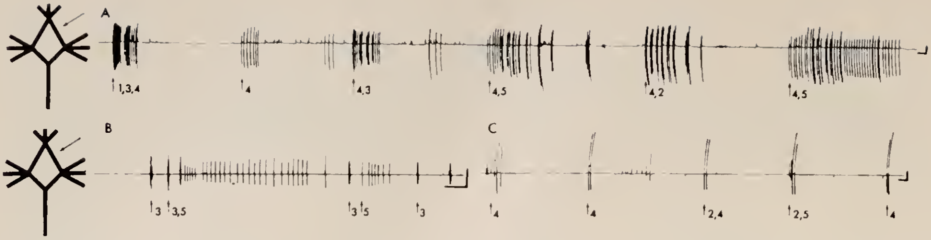

FIGURE 8. Types of hydranth pulse (HP) activity and associated behavioral correlates in *Corymorpha.* (A–C) Upward pointing arrows beneath the record indicate the point at which the behavior associated with the following numbers began: (1.) distal tentacles flare upward; (2.) distal tentacles flare downward; (3.) proximal tentacles simultaneously sweep inward around the proboscis; (4.) tip of the proboscis pulls downward; (5.) whole animal contracts. Vertical scale equals 1 mV; horizontal equals 5 sec.

segments of the stalks. Bursts of this length are often seen in intact stalks, again suggesting that pulses are normally triggered basally. The site of pulse initiation does sometimes shift within a burst (Fig. 10). *Corymorpha* does not have a distinct class of neck pulse as does *Tubularia;* but the stalk pulses of *Corymorpha* appear to have the same functional correlates as the neck pulses of *Tubularia.*

Hydranth pulses (*HP* > *100*). Hydranth pulses may occur alone, in pairs, or in bursts of varying length (Fig. 8). The most common pattern originating within the hydranth appears to be two to six pulses in quick succession at fairly regular intervals. (Fig. 8C). There are also many longer bursts originating either within the hydranth or the stalk (Fig. 8A, B). Waveforms vary greatly both within and between animals.

The behavioral correlates of hydranth pulses vary greatly from animal to animal. It is frequently difficult to discern any clearcut behavioral correlate of the commonly occuring regular pairs of pulses, although in some cases a wave of peristalsis passes down the proboscis and ends in a rather abrupt downward jerk of the proboscis coincident with each short burst of electrical activity. A complete behavioral response to a burst of hydranth pulses appears to be a simultaneous inward flexion of all proximal tentacles, associated with proboscis withdrawal but the observed response is frequently only some portion of this complete response. Thus, there may be a contraction with proximal tentacle flexion, or only some of the proximal tentacles may flex inward, or the proximal tentacles may flex inward only part way.

Proximal tentacle pulses (*PTP* > *100*). Each proximal tentacle has independent pacemakers that may generate pulses singly or in bursts. Frequently tentacle bursts are triggered by bursts of stalk or hydranth pulses, and when such triggering occurs the same burst pattern is usually recordable in all proximal tentacles along with occasional spontaneous activity. However, in other instances electrical activity in some or all of the tentacles may show no relation to that elsewhere in the animal.

It is likely that the site of PTP initiation varies from time to time and animal to animal. In most cases pulses recordable at the tip of a proximal tentacle and

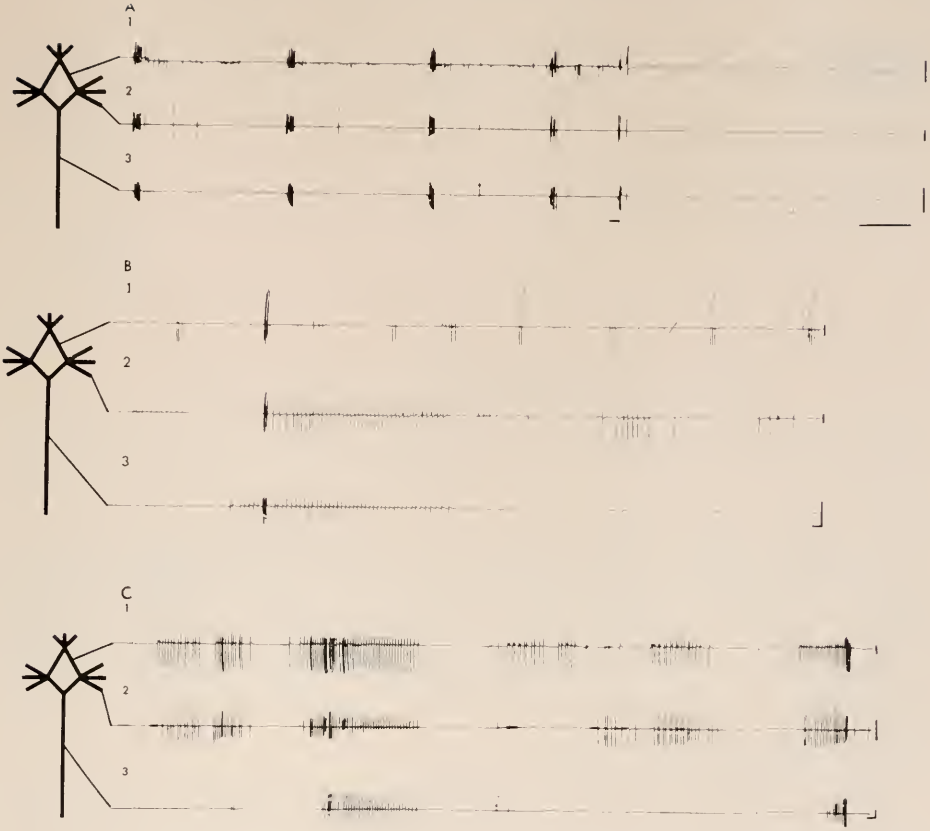

Figure 9. Relationships between electrical activity in the various pulse systems of *Corymorpha.* Vertical scale equals 1 mV; horizontal equals 5 sec, except that on the right in A which equals 0.1 sec.

not triggered from outside it fail to reach its base and are therefore not recorded in immediately adjacent proximal tentacles.

When recording from the whole animal there is often no apparent behavioral correlate of proximal tentacle pulses, although in some instances the tentacle is clearly flexing against the resistance of the electrode. This inactivity, however, appears to be an artifact of the recording conditions, since obvious flexions or contractions of the tentacles associated with electrical activity are observed in relatively less restricted isolated tentacles.

Distal tentacle pulses (*DTP* > *15*). Each distal tentacle has its own independent pacemaker, a fact readily apparent only after excision since there is little spontaneous electrical activity in attached tentacles. Distal tentacle pulses may occur singly or in bursts and usually follow activity in the proboscis. Therefore, the electrical activity in all distal tentacles is normally quite similar, in contrast to the situation in the proximal tentacles. There is no behavioral correlate of distal tentacle pulses recorded from the whole animal. As in the case of the proximal

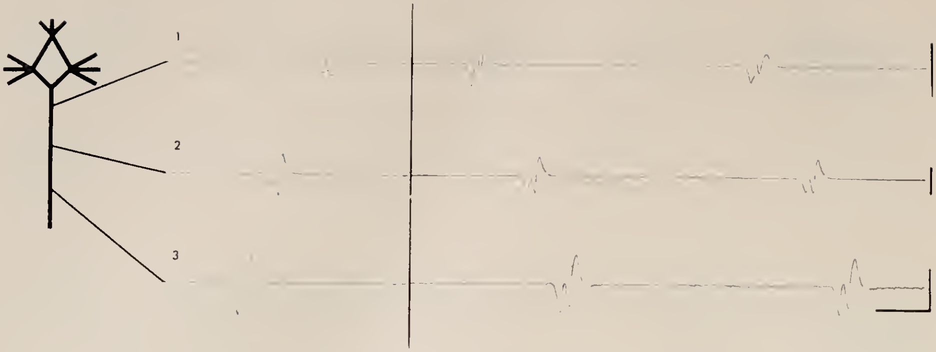

Figure 10. Lead shift during a burst. Pulses are consecutive. Vertical scale equals 500 μV; horizontal equals 0.1 sec.

tentacles, however, this appears to be an artifact caused by restriction by the electrode.

Gonophore peduncle pulses (*GPP > 10*). Gonophore peduncle pulses may occur singly, but more often they occur as part of a burst, triggered from the proboscis. During a concert with stalk contraction it appears that the gonophore peduncles sometimes contract at about the same time as the proximal tentacles move inward around the hydranth. Spontaneous contraction of individual peduncles also occurs.

Interactions between pulse systems

Details of the interactions between spontaneous pulse systems are complex and somewhat variable, but certain valid generalizations can be made. Stalk bursts normally result in activity which can be recorded everywhere in the animal (Fig. 9A). During a stalk burst in an intact animal the bursts occurring both in the proximal tentacles and in the hydranth normally appear to be driven directly by stalk activity, but in some cases (Fig. 9B) only the proximal tentacles are driven. Proximal tentacle bursts are also often driven directly from the hydranth without stalk participation (Fig. 9C).

Since stalk bursts sometimes appear to be initiated by electrical activity in the hydranth the only generalization which appears to be valid is that a burst occurring

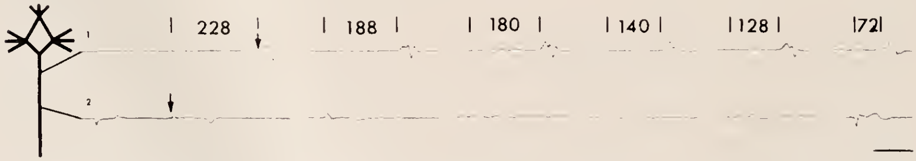

Figure 11. Evidence for a shifting pacemaker or for changing conduction velocity during a burst. The numbers above the record indicate the latency (msec) between the beginning of a single event as recorded at two places on the stalk. These intervals were measured as shown by the arrows associated with the first pulse. No vertical calibration was recorded.

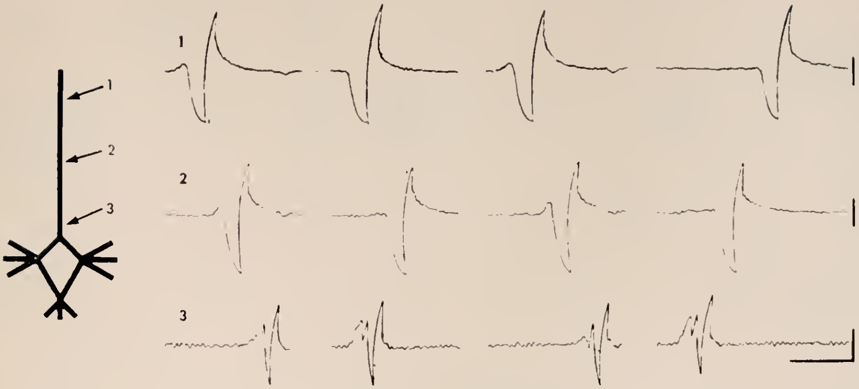

FIGURE 12. Records which can most easily be explained by hypothesizing simultaneous independent activation of more than one pacemaker. Vertical scale equals 500 μV; horizontal equals 0.1 sec. Sections were removed from the record (dotted lines) to allow reproduction.

in the stalk, regardless of its point of initiation, will usually also be recorded in the hydranth and proximal tentacles. In some cases the lead (triggering pulse) shifts back and forth between the hydranth and stalk or apparently between different parts of the stalk (Fig. 10), possibly indicating that the conducting system is unstable and can be triggered by small disturbances at many sites.

Successive pulses of a burst sometimes appear to show a marked change in velocity (Fig. 11). One alternative to an actual change in velocity is that several pulse initiation sites have been activated in different regions and are running independently of each other. Figure 12 shows a series of records which can apparently only be explained by hypothesizing independent activation of multiple sites of pulse initiation.

Electrical activity and movement of isolated parts of Corymorpha

In an isolated part electrical activity is often absent or at least reduced immediately following excision, but it usually returns within several minutes. Frequently there is a change in the pulse pattern. For example, the pulses may become more or less grouped into bursts; but it is impossible to attribute such changes unequivocally to excision. Figure 13A shows activity before and after the excision (a) of a proximal tentacle from the hydranth, then (b) of the hydranth from the stalk.

A much more direct correlation between electrical activity and movement is apparent in excised parts than in the whole animal. In most proximal tentacles there is nearly a one to one correlation between movements of the tentacles and single pulses, although sometimes there are pulses without obviously associated movements, and during bursts there may be a movement only on the first pulse. In distal tentacles movement appears to be correlated with each pulse, but many flexions lack apparent associated electrical activity. Excised proximal tentacles are capable of producing complex bursts (Fig. 13B, C) probably indicating simultaneous activation of more than one pacemaker. In excised gonophore peduncles most

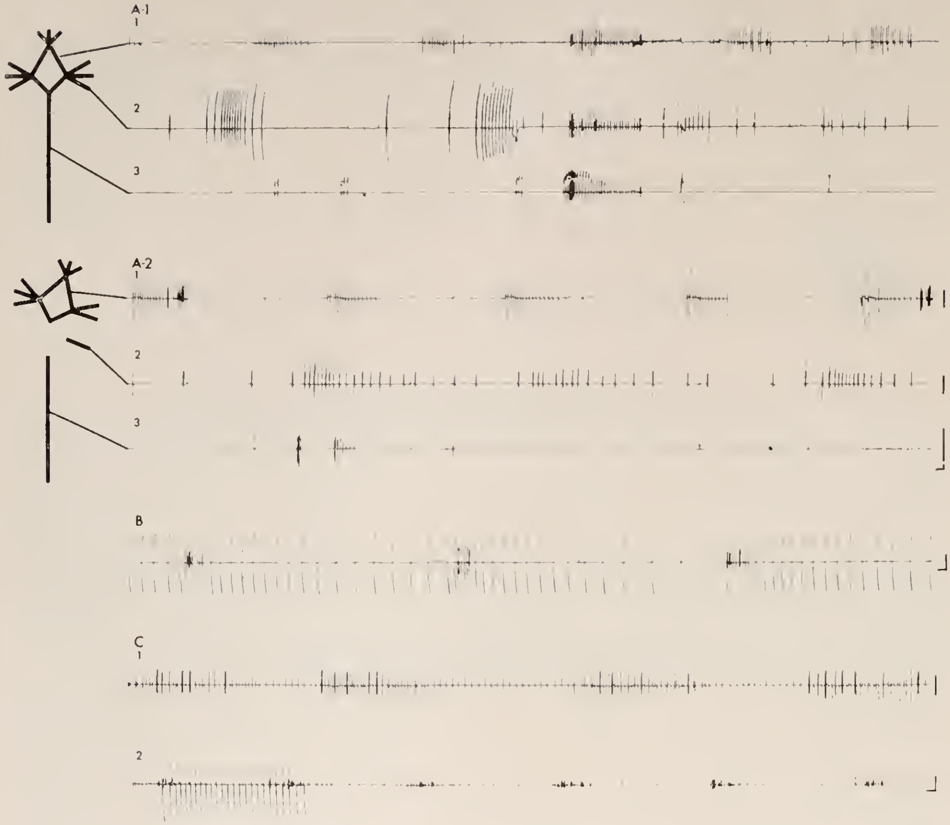

FIGURE 13. Excision experiments; (A.) electrical activity in various parts of a *Corymorpha* before (A–1) and after (A–2) their separation from the rest of the animal. (B.) An excised proximal tentacle is capable of producing bursts. (C.) More bursts produced by excised proximal tentacles. These records were made at 27° C. Records B and C2 are from the same tentacle. The excised proximal tentacle in A–2 was held by the tip; those in B and C were held by the cut basal ends. Vertical scale equals 2 mV; horizontal equals 5 sec.

activity appears to occur in bursts but there is no clear association between electrical activity and movement.

In excised hydranths the proximal tentacles continue to flare inward at the start of hydranth bursts, and the proboscis contracts during the burst if this behavior was initially present in the whole animal. Hydranth bursts often occur at quite regular intervals following excision, but not enough records were obtained to determine whether the apparent increase in regularity is significant. Isolated stalks contract either on the first pulse of a stalk burst or throughout the burst. Bursts of stalk pulses often come quite regularly.

In summary, excision experiments confirm the experiments on intact animals in indicating that there are at least five sets of pacemakers; namely, those in the stalk, hydranth, proximal tentacles, distal tentacles, and gonophore peduncles.

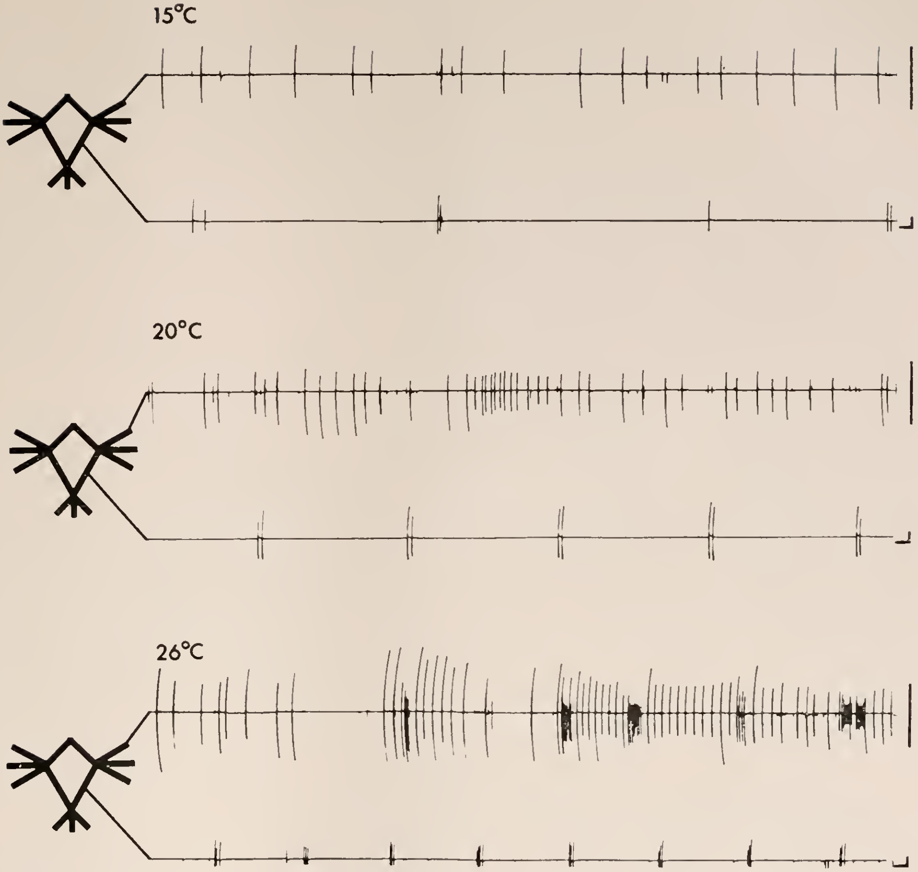

FIGURE 14. Effect of temperature on electrical activity in an excised hydranth of *Corymorpha.* All records are from the same hydranth. Temperatures are accurate to ±1° C. Vertical scale equals 2 mV; horizontal scale equals 5 sec.

Effects of temperature on spontaneous electrical activity

Within the investigated range from 10 to 30° C pulse frequency increases with increasing temperature. Figure 14 shows the effect of temperature on electrical activity in a proximal tentacle and the proboscis of an excised hydranth. At temperatures in the neighborhood of 28° C animals sometimes generate a steady stream of high frequency pulses. Up to about this temperature the animals become more active, but beyond it they stand rather quietly. Pulse amplitude generally decreases below approximately 15° C and above about 28° C. Rapid temperature transitions, especially upwards, often cause an animal to become extremely active electrically.

DISCUSSION

As seen by comparison with the investigations of Josephson and Mackie (1965), the spontaneous pulse systems of *Tubularia* and *Corymorpha* are very much alike

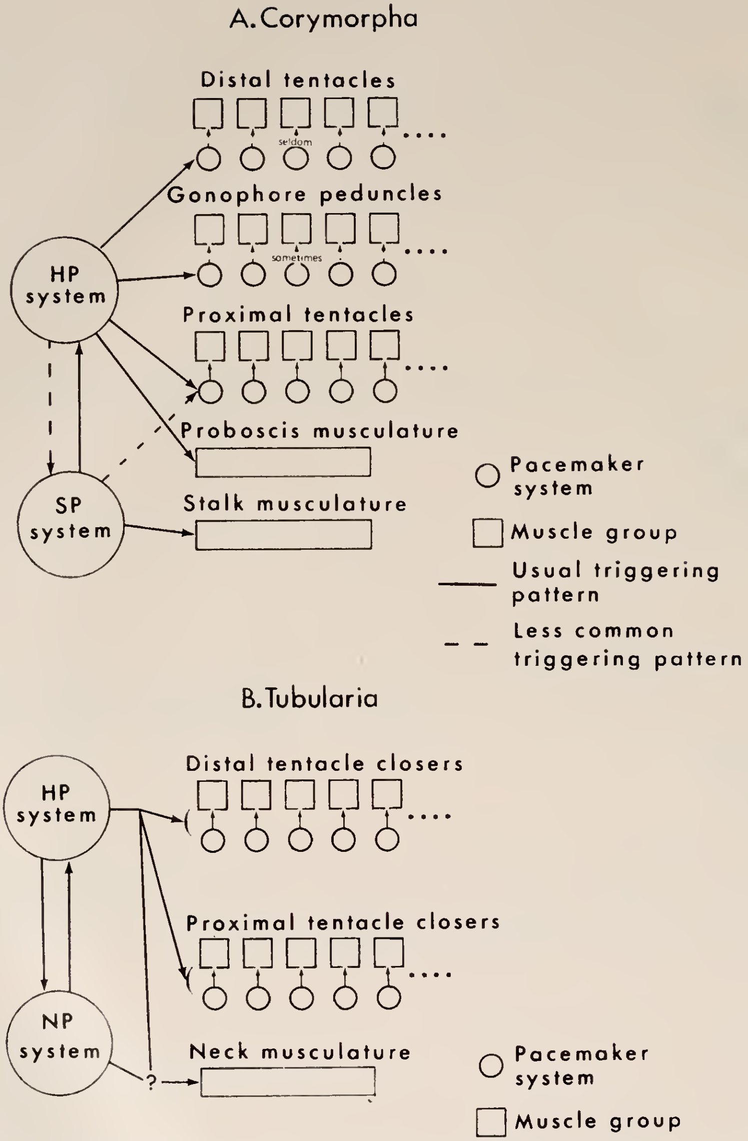
A. Corymorpha
Distal tentacles
seldom
Gonophore peduncles
sometimes
HP system
Proximal tentacles
Proboscis musculature
SP system
Stalk musculature
Pacemaker system
Muscle group
Usual triggering pattern
Less common triggering pattern
B. Tubularia
HP system
Distal tentacle closers
Proximal tentacle closers
NP system
Neck musculature
?
Pacemaker system
Muscle group

FIGURE 15. Comparison of interactions between pacemakers and effector systems in *Corymorpha* and *Tubularia;* (A.) *Corymorpha* (diagram modeled after that of Josephson and Mackie, 1965, for *Tubularia* which constitutes B of this figure), (B.) *Tubularia* (Josephson and Mackie, 1965).

in most respects. Pulse durations, waveforms, and amplitudes are all relatively similar in the two organisms. Stalk pulses in *Corymorpha* and neck pulses in *Tubularia* occur in relatively long bursts, although single pulse activity is quite rare in *Corymorpha* stalks and common in necks of *Tubularia*. Hydranth activity in both animals often occurs in bursts of two to six pulses except when triggered by SPs (*Corymorpha*) or NPs (*Tubularia*). Proximal and distal tentacles and gonophore peduncles all have independent pacemakers although much of their electrical activity is triggered elsewhere in the animal. The pulse systems of both organisms are arranged in similar hierarchies (Fig. 15).

There are two significant differences between the spontaneous pulse systems of *Corymorpha* and *Tubularia* as they are diagrammed in Figure 15. First, proximal tentacle pulses are sometimes directly driven by stalk pulses in *Corymorpha*, but there is no indication that the NPs of *Tubularia* can perform a similar function. Secondly, the brackets enclosing both pacemakers and muscle groups in the distal tentacles of *Tubularia* indicate that the enclosed pacemakers and muscle groups are tightly coupled. In *Corymorpha* most hydranth pulse activity is recorded both in distal tentacles and in gonophore peduncles, but there is often no visible response associated with electrical activity in these structures.

There is considerable inter-individual variability in the relationship between electrical activity and behavior. This variability can be disconcerting, but after working with large numbers of either *Tubularia* or *Corymorpha* it becomes obvious that there is a general relationship, encompassing all individual variations, between electrical activity and behavior. For example, the extent of concert activity associated with hydranth pulses varies from an almost imperceptible contraction of the proboscis or a small inward flexion of the proximal tentacles to a violent, simultaneous oral movement of all the proximal tentacles associated with readily visible contraction of the proboscis. Concerts associated with stalk contraction are, in general, more vigorous than those initiated within the hydranth itself. There is also "loose coupling" (Josephson and Mackie, 1965) between stalk pulses and hydranth pulses, as exemplified by the failure of some stalk bursts to trigger concert behavior in some animals.

In contrast to this high degree of inter-individual variability the behavior of each animal is quite consistent. That is, once electrical activity and behavior of a given animal have been stimultaneously observed for about 20 minutes, it usually becomes possible to describe the animal's behavior quite accurately from the electrical record alone.

Temperature has a marked effect on pulse generation frequency in *Corymorpha*, but, since the cellular elements responsible for this function have yet to be identified unequivocally the mechanism of this effect remains unknown. Rushforth (1971) and Rushforth and Burke (1971) have investigated pacemaker activity in *Hydra* and much of their discussions of pacemaker properties and possible models is also applicable to *Corymorpha*.

In *Corymorpha* the various pulse types are not distinguishable on the basis of waveform, amplitude, or duration. The average amplitude of distal tentacle pulses is considerably larger than that of other pulse types, but this is presumably an artifact of the much finer electrodes used to avoid entirely enveloping a distal tentacle. Also, it appears that all parts of the animal, with the possible exception of the perisarc-covered base of the stalk, are capable of pulse generation. Bearing this in mind, it might be argued that the pulse categories which have been set up are purely arbitrary. However, each of the five categories constitutes a demonstrable functional unit, which appears to be adequate justification for its establishment.

This account of the relation between electrical activity and behavior in *Corymorpha* leaves much complex behavior unexplained in terms of electrical activity. For instance, some distal tentacle movements and opening and closing of the mouth appear to occur without associated electrical activity, as does peristalsis of the proboscis, which is, however, often temporarily terminated by a contraction associated with hydranth pulses. Although the inhibition of concert and bowing behavior by a current suggests a sophisticated receptor-effector system, the elements of this system, and their mode of interaction, remain unknown. The same can be said of the mechanisms co-ordinating and controlling both regurgitation and the localized stalk contraction required to produce bowing. In general, the electrical activity which has been described here appears to be associated with contraction of the longitudinal musculature, although, as previously mentioned, there have been some animals and excised parts where such contraction was not visible.

Following Mackie's (1965) demonstration of epithelial conduction in siphonophores almost all investigators have regarded the relatively large spontaneous pulses which are recordable from many hydroids as being non-neural in origin. The main arguments supporting this hypothesis are the following: (1) the pulses are much too large and easily recordable with suction electrodes to originate in the fine neurons of the nerve net; (2) many types of pulses have a wide-spread distribution within the part of the animal where they occur; and (3) the duration of the pulses is greater than that of nerve impulses, with the possible exception of impulses in a few neurosecretory cells.

If the pulses recordable from *Corymorpha* are assumed to be nonneural, the role of the nervous system, the relation between electrical activity in the nervous system and that in the epithelial system, and any causal relationships between electrical activity in the two systems and behavior all remain to be elucidated. These relationships are considered in more detail in the following paper.

I thank Dr. James Case, Dr. James Morin and Dr. R. K. Josephson for helpful discussions during both research and writing and Dr. Larry Friesen for drawing Figure 1. The research was supported by an NDEA Title IV Predoctoral Fellowship and by PHS Grant NS 08599 to Dr. J. F. Case. This paper is based on part of a thesis presented to the Department of Biological Sciences, University of California, Santa Barbara, in partial fulfilment of the requirements for the Ph.D.

SUMMARY

1. There is a clear association between certain aspects of the behavior of *Corymorpha palma* and electrical activity of five interacting pulse systems located in the stalk, hydranth, proximal tentacles, distal tentacles, and gonophore peduncles.

2. Stalk pulses normally occur in bursts of 10 to 30, and are associated with contraction of the longitudinal musculature of the stalk. They apparently may originate anywhere on the stalk, but are usually initiated in its basal half.

3. Hydranth pulses occur in bursts of varying lengths, the most common consisting of 2–6 pulses. These pulses are associated with a "concert" which varies greatly in intensity from animal to animal. A full concert involves contraction of the proboscis as the proximal tentacles come in around it, though some animals show nothing more than a twitch of the proximal tentacles or a barely perceptible contraction of the proboscis.

4. Proximal and distal tentacle pulses may occur singly or in bursts which are usually triggered from outside the tentacle. While the tentacle is attached to the animal there is sometimes no obvious behavioral correlate of these pulses; but with excised tentacles there is usually a flexion or slight contraction associated with each pulse or burst of pulses.

5. Gonophore peduncle pulses may occur singly or in bursts which are usually triggered from outside the peduncle. In some cases there is no obvious behavioral response to these pulses, while in others there is a contraction of the ectodermal musculature.

6. The pulse systems are arranged in a coupled hierarchy with stalk bursts normally triggering activity everywhere else in the animal, either directly or by way of the hydranth pulse system. This triggering results in concerts associated with stalk contraction; such concerts occur either irregularly or at fairly regular intervals of several minutes in undisturbed animals. Hydranth pulses trigger pulses in both sets of tentacles, resulting in concerts. Excision experiments were used to clarify these relationships between the various pulse systems.

7. Temperature has a marked effect on pulse activity. Proximal tentacle pulses and hydranth pulses are much more frequent at high temperatures and are less frequent at low temperatures over the range 10–30° C.

8. The spontaneous pulse systems of *Tubularia* and *Corymorpha* are similar in many respects.

9. The pulse systems of *Corymorpha* cannot be differentiated on the basis of pulse duration, amplitude, or waveform.

10. Much of the more complex behavior of *Corymorpha* cannot be explained in terms of recordable electrical activity. The mechanisms by which this behavior is controlled remain unknown.

LITERATURE CITED

CHAPMAN, G., AND R. L. PARDY., 1972. The movement of glucose and glycine through the tissues of *Corymorpha palma* Torrey (Coelenterata, Hydrozoa). *J. Exp. Biol.,* **56**: 639–645.

GATENBY, J. B., AND T. S. PAINTER, 1937. *The Microtomist's Vade-Mecum: A Handbook of the Methods of Animal and Plant Microscopic Anatomy.* Blakiston, Philadephia, 784 pp.

HYMAN, L. H., 1940. *The Invertebrates: Protozoa through Ctenophora.* McGraw-Hill, New York, 726 pp.

JOSEPHSON, R. K., 1961a. Colonial responses of hydroid polyps. *J. Exp. Biol.,* **38**: 559–577.

JOSEPHSON, R. K., 1961b. Repetitive potentials following brief electrical stimuli in a hydroid. *J. Exp. Biol.,* **38**: 579–593.

JOSEPHSON, R. K., AND G. O. MACKIE, 1965. Multiple pacemakers and the behaviour of the hydroid *Tubularia. J. Exp. Biol.,* **43**: 293–332.

JOSEPHSON, R. K., AND J. UHRICH, 1969. Inhibition of pacemaker systems in the hydroid *Tubularia. J. Exp. Biol.,* **50**: 1–14.

MACKIE, G. O., 1965. Conduction in the nerve-free epithelia of siphonophores. *Amer. Zool.,* **5**: 439–453.

MACKIE, G. O., 1968. Electrical activity in the hydroid *Cordylophora. J. Exp. Biol.,* **49**: 387–400.

MORIN, J. G., AND I. M. COOKE, 1971a. Behavioural physiology of the colonial hydroid *Obelia.* I. Spontaneous movements and correlated electrical activity. *J. Exp. Biol.,* **54**: 689–706.

MORIN, J. G., AND I. M. COOKE, 1971b. Behavioural physiology of the colonial hydroid *Obelia.*

II. Stimulus-initiated electrical activity and bioluminescence. *J. Exp. Biol.*, **54**: 707–721.

Parker, G. H., 1917. The activities of *Corymorpha*. *J. Exp. Zool.*, **24**: 303–331.

Parker, G. H., 1919. *The Elementary Nervous System*. J. B. Lippincott, Philadelphia, 299 pp.

Parmentier, J., and J. Case, 1973. Pharmacological studies of coupling between electrical activity and behaviour in the hydroid. *Tubularia crocea* (Agassiz). *Comp. Gen. Pharmacol.*, **4**: 11–17.

Passano, L. M., and C. B. McCullough, 1962. The light response and rhythmic potentials of *Hydra*. *Proc. Nat. Acad. Sci.*, **48**: 1376–1382.

Passano, L. M., and C. B. McCullough, 1963. Pacemaker hierarchies controlling the behaviour of hydras. *Nature*, **199**: 1174–1175.

Passano, L. M., and C. B. McCullough, 1964. Coordinating systems and behaviour in *Hydra*. I. Pacemaker system of the periodic contractions. *J. Exp. Biol.*, **41**: 643–664.

Passano, L. M., and C. B. McCullough, 1965. Coordinating systems and behaviour in *Hydra*. II. The rhythmic potential system. *J. Exp. Biol.*, **42**: 205–231.

Rushforth, N. B., 1971. Behavioral and electrophysiological studies of *Hydra*. I. Analysis of contraction pulse patterns. *Biol. Bull.*, **140**: 255–273.

Rushforth, N. B., and D. S. Burke, 1971. Behavioral and electrophysiological studies of *Hydra*. II. Pacemaker activity of isolated tentacles. *Biol. Bull.*, **140**: 502–519.

Torrey, H. B., 1902. The hydroida of the Pacific Coast of North America. *Univ. Calif. Publ. Zool.*, 1: 1–104.

Torrey, H. B., 1904a. Biological studies on *Corymorpha*. I. *C. palma* and environment. *J. Exp. Zool.*, 1: 395–422.

Torrey, H. B., 1904b. The hydroids of the San Diego Region. *Univ. Calif. Publ. Zool.*, **2**: 1–43.

Torrey, H. B., 1905. The behavior of *Corymorpha*. *Univ. Calif. Publ. Zool.*, **2**: 333–340.

Torrey, H. B., 1907. Biological studies on Corymorpha. II. The development of *C. palma* from the egg. *Univ. Calif. Publ. Zool.*, **4**: 253–298.

Torrey, H. B., 1910a. Biological studies on *Corymorpha*. III. Regeneration of hydranth and holdfast. *Univ. Calif. Publ. Zool.*, **6**: 205–221.

Torrey, H. B., 1910b. Note on geotropism in *Corymorpha*. *Univ. Calif. Publ. Zool.*, **6**: 223–224.

Torrey, H. B., 1910c. Biological studies on *Corymorpha*. IV. Budding and fission in heteromorphic pieces and the control of polarity. *Biol. Bull.*, **19**: 280–301.

Reference: *Biol. Bull.*, **145**: 243–264. (October, 1973)

ELECTRICAL ACTIVITY AND BEHAVIOR IN THE SOLITARY HYDROID *CORYMORPHA PALMA*.

II. CONDUCTING SYSTEMS

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Attempts to understand the relationship between various types of cnidarian conducting systems and between electrical activity in these systems and behavior have been based largely on three general approaches; (1) surgical alteration of the system, (2) application of drugs or alteration of the ionic environment, and (3) electrical stimulation. The use of these methods on hydrozoan and scyphozoan medusae and on anthozoans has resulted in considerable advances in understanding the physiological basis of their behavior. However, with the exception of *Hydra* it has not been possible to apply all of these techniques to individuals of any one species of hydrozoan polyp because of small size and the presence of a perisarc. We therefore decided to apply these techniques to *Corymorpha,* which, because of its large size and reduced perisarc, appeared well suited to an investigation utilizing all of these approaches.

Electrical and behavioral responses to electrical stimulation have been examined in the hydroids *Hydractinia* (Josephson, 1961) *Cordylophora* (Mackie, 1968), *Tubularia* (Josephson, 1961, 1965; Josephson and Uhrich, 1969) *Hydra* (Josephson, 1967; Josephson and Macklin, 1967, 1969; Kass-Simon, 1972) and *Obelia* (Morin & Cooke, 1971). In *Tubularia* Josephson (1965) found evidence for three conducting systems: (1) a distal opener system producing a small pulse conducted at 15 cm/sec and associated with a downward flaring of the distal tentacles; (2) a triggering system which triggers the type of pulses that normally appear spontaneously, conducts at approximately 17 cm/sec, and has no electrical correlate; and (3) a labile slow system producing propagated potentials which travel at about 6 cm/sec in the stalk.

There is a long history of investigations involving the application of drugs and ions to medusae (see Mackie and Passano, 1968), while somewhat less work has been done on anthozoans (see Ross, 1960a, 1960b), and relatively little on hydrozoan polyps. Parmentier and Case (1973, page 17), working on the hydroid *Tubularia* found that: "The sympathomimetic compound ephedrine induces steady rates of impulse production in the spontaneous pulse system regardless of the pre-treatment discharge pattern without disrupting behavioral responses and coordinated epitheliomuscular output." From these results they concluded that the epithelial system had little direct relationship to localized behavioral activation, although they felt that it might serve to coordinate more symmetrical output patterns throughout the organism. In view of these results we decided to test the effect of ephedrine and related sympathomimetic drugs on electrical activity and behavior in *Corymorpha*.

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Bullock (1943), investigating the effects of alteration in the ionic environment on the neuromuscular systems of medusae, found that excess Mg^{++} first depresses myo-neural junctional facilitation and then neuro-neural junctional facilitation. In *Tubularia* Josephson (1965) was able to separate electrically triggered distal opening system pulses (DOSP) from the following slow wave by application of excess Mg^{++}. He interpreted this result as indicating that the DOSP was associated with nerve activity while the slow wave was a muscle action potential, with excess Mg^{++} blocking the link between the two. These results led us to the idea for the Mg^{++} bridge experiment described in this paper.

Mackie (1968) made the interesting discovery that tetrodotoxin, which selectively interferes with the early transient sodium-conductance increase in lobster (Narahashi, Moore, and Scott, 1964) and squid axons (Nakamura, Nakajima, and Grundfest, 1965), had no effect on electrical activity or behavior of *Cordylophora* over periods of more than an hour. This finding, if generally applicable to coelenterates, is of considerable interest since it may indicate that their pacemakers and conducting systems differ from those of most organisms by not being Na^{+} dependent. This experiment was therefore repeated on *Corymorpha*.

MATERIALS AND METHODS

Electrical stimulation

In the majority of the experiments involving electrical stimulation a *Corymorpha* was laid out horizontally on a sheet of cork and held in place by Pt-Ir staples across the stalk. Stimulation was normally basal; but stimuli were also applied near the neck, or at both ends of the stalk, either simultaneously or with varying interstimulus delays. Animals standing upright on sand were also stimulated to establish whether the position of the animal or the restraint of the staples produced any effect on response to stimulation.

Stimuli were applied from Grass S4 and S48 stimulators through Grass Stimulus Isolation Units. The stimulating electrodes used in most experiments were either Pt-Ir (0.2 mm) or Ag-AgCl (1.6 mm) insulated to the tips with "Insulex" or silicon rubber. In a few experiments a "Tygon" suction electrode (i.d. 1.6 mm), similar to those used for recording, was used for stimulation with a Pt-Ir indifferent electrode in the bath. Electrode polarity was periodically reversed to avoid polarization. Unless otherwise noted stimuli were 5 msec monophasic square waves of varying voltages. The pulse duration was chosen arbitrarily because it appeared to be adequate to obtain a full response from most animals. Stimulation was increased from low voltages fairly rapidly until an electrical or behavioral threshold was reached.

Unless otherwise specified recording electrodes, conditions and equipment were as described in the previous paper. Interelectrode distances were measured using dividers or a small ruler. When these distances changed noticeably during the course of a burst the distances before and after stimulation were averaged. Measurements were made often enough so that the variability introduced by averaging was very small compared to the apparent normal variability of the system. Methodological details of some further experiments are discussed in conjunction with the results.

Drugs

All drug solutions were made up in filtered sea water (5 μ pore size filter) with the final pH adjusted within the range of pH 7.0–8.0. All experiments were run at 20 ± 1° C. Drug concentrations and numbers of replicates are summarized in Table I. Several types of experimental chambers were used, with the exact size and shape depending on the size of the preparation and on the details of the experiment. Solutions were withdrawn and replaced using two syringes with a capacity greater than that of the bath. Immediately preceding solution changes the electrodes, and the tissue to which they were attached, were lowered as near to the bottom of the bath as possible, thus considerably reducing the chances of the electrode detaching during the change of solutions. The fluid to be replaced was then withdrawn almost completely, resulting in only a slight dilution of the fluid being added. The general procedure used in these experiments was (a) to record from the preparation in sea water for a minimum of 15–20 minutes, (b) to apply the drug being tested and record for 15–20 minutes, then (c) to replace the drug with sea water and record another 15–20 minutes.

For an experiment involving tetrodotoxin a sheet of stalk tissue was arranged on a thick pad of porous cloth in an effort to provide the drug with free access to both sides of the sheet. Due to the low level of spontaneous activity in stalk tissue, the response to a single electrical stimulus given every 100 seconds during periods of observation was used as an additional assay for effects of the drug.

Inorganic ions

Experiments involving altered ionic composition of the bathing solutions included (a) application of Moore's Ca^{++}-free sea water (Cavanaugh, 1956) which has the following ionic composition (g/liter), NaCl, 25.48; KCl, 0.72; $MgCl_2 \cdot 6H_2O$, 6.94; $MgSO_4 \cdot 7H_2O$, 4.11; and (b) application of various concentrations of excess Mg^{++} in the form of $MgCl_2$ solutions. For some of the experiments on the effects of $MgCl_2$ the isolated stalk of a large *Corymorpha* was draped across three compartments cut into a block of wax and held in place by staples passing over the stalk. There was some leakage of solutions along the stalk over the partitions separating the compartments, but this leakage had no apparent effect during the relatively short time required for the experiments. A suction electrode was used to record from the portion of the stalk in each compartment, and stimulation was via two wires placed on opposite sides of the base of the stalk. A precondition for proceeding with the experiment was that five successive stimuli at 1-minute intervals had to produce one or more pulses recorded on each of the electrodes. Once this precondition had been met, the sea water in the center compartment was replaced with isotonic $MgCl_2$ and stimulation at 1-minute intervals was continued. The length of time that the $MgCl_2$ remained in the central chamber depended on the responses of the individual animal; but the $MgCl_2$ was eventually replaced by repeated changes of sea water. The sea water in the first and third

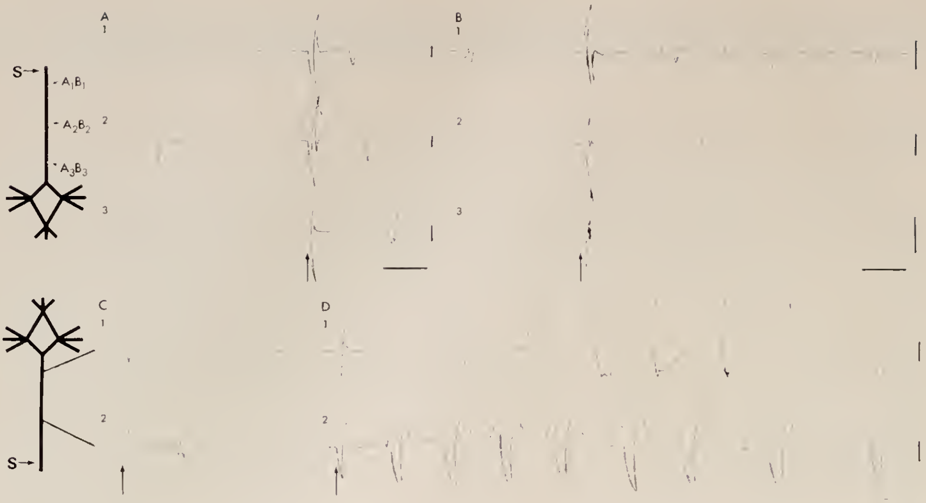


FIGURE 1. Some characteristic responses of the triggered pulse system in *Corymorpha.* (A and B.) Both records, from two different animals, show consecutive pulses, the first spontaneous and the others triggered. Note that in A the pulses appear similar although they are traveling in opposite directions; (C and D.) effect of increased stimulating voltage on electrical response. Sections were removed from some of the records (dotted lines) to allow reproduction. The large S and arrow adjacent to each diagram of *Corymorpha* in this and all other figures in this paper indicate the point at which the stimulus was applied. Stimulus artifacts in the records in this and all other figures in this paper are marked with upward-pointing arrows. Vertical scales of A and B are 500 μV; those of C and D are 50 μV. Horizontal scale equals 0.1 sec. Portions of the records were retouched for reproduction.

chambers was also replaced, due to some leakage of $MgCl_2$ from the center compartment into the others. Single stimuli were then given at irregular intervals to observe the progress of recovery from $MgCl_2$.

RESULTS

Behavioral responses of the unrestrained animal.

The responses to stimulation of a proximal tentacle of an unrestrained animal with successive single pulses of increasing voltage are similar to those described by Parker (1917): first only the stimulated tentacle responds; then adjacent tentacles; then the distal tentacles and the proboscis, which may bend toward the stimulated tentacle; and finally a stalk contraction may be produced.

Electrical and behavioral responses of the restrained animal.

Characteristics of the triggered pulse system (TPS) in response to stimuli of differing voltages. The pulses most commonly generated in the stalk in response to electrical stimulation appear to be identical to spontaneously occurring pulses (Fig. 1A, B). However, to facilitate discussion triggered pulses will be called TPs and the system will be called the TPS (triggered pulse system). Frequently, especially at low stimulating voltages, the electrical response to stimulation is a

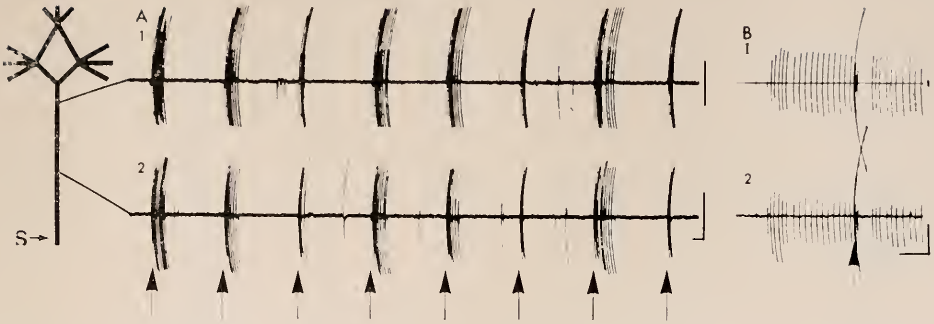


FIGURE 2. Excitation and inhibition produced by single electrical stimuli; (A.) intact animal, (B.) a sheet of stalk tissue. Vertical scale equals 100 μV; horizontal scale equals 5 sec.

single small pulse travelling along the stalk in a non-decrementing fashion (Fig. 1C). In the majority of cases where this is the sole electrical response there is either no visible reaction or there is a slight overall contraction.

At slightly higher stimulating voltages a burst of pulses is usually produced (Fig. 1D). Associated with this burst there is usually a more violent contraction and there may be a simultaneous oral flexion of all proximal tentacles sometimes accompanied by an aboral flaring of the distal tentacles. Some animals show a fairly clear-cut threshold for burst initiation, while in others this threshold varies. Both the intensity of contraction and the length of a stimulus-initiated burst appear to depend on the strength of the stimulus. Stimulation normally produces bursts of electrical activity (Fig. 2A); but it can also interrupt ongoing spontaneous electrical activity (Fig. 2B). Fig. 3 shows several bursts all produced by the same animal in response to single electrical stimuli.

Conduction velocity in the TPS. Apparent conduction velocity in the TPS varies both within an animal over time and, to an even greater extent, between animals. There is no apparent difference in the conduction velocity as measured between two points on the stalk and between one point on the stalk and another on the hydranth. The average conduction velocity between two points on the stalk determined from a minimum of five measurements on each of ten animals was 15.7 cm/sec. Individual averages varied from 10.9 cm/sec to 21.2 cm/sec. This average value is quite close to the average spontaneous pulse conduction velocity of 15.9 cm/sec which appears to strengthen the case for the identity of these two systems. However, the use of average values may be misleading. Records sufficient to allow calculation of conduction velocities for both triggered and spontaneous pulses in the same preparation were obtained from six animals. In four of these there was little difference (±1 cm/sec) in velocity. The other two gave the following averages for consecutive sets of spontaneous and triggered bursts. (1) spontaneous 23.8 cm/sec, triggered 16.9 cm/sec, spontaneous 26.2 cm/sec; (2) spontaneous 14.0 cm/sec, triggered 10.5 cm/sec, spontaneous 14.0 cm/sec.

Tests for polarization in the TPS. To check whether the TPS was polarized, the stimulating electrodes were placed in the neck region of a *Corymorpha* with the recording electrodes farther down the stalk. The average conduction velocity

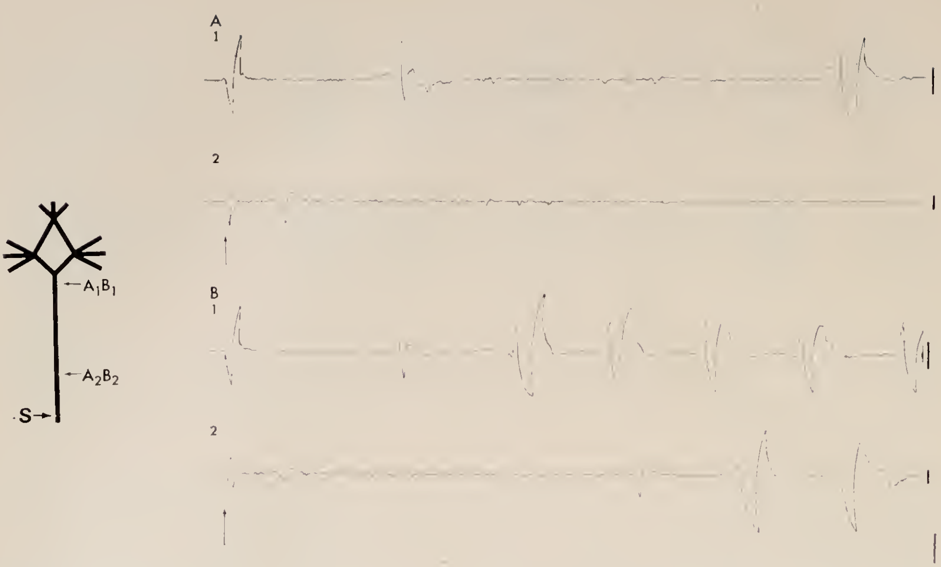


FIGURE 3. Variability in pulse patterns of one *Corymorpha* in response to single electrical stimuli; (A.) large pulses interspersed among a series of much smaller pulses; (B.) a single triggered pulse traveling distally has apparently initiated a burst of activity originating from the other end of the stalk. The significance of the apparent differences in initial polarity of large and small pulses is unknown. Stimulating and recording electrodes were in the same positions for each record. Stimulus artifacts in the records are marked with upward-pointing arrows. Vertical scale equals 50 μV; horizontal scale equals 0.1 sec.

determined from a minimum of five measurements on each of eight animals was 15.2 cm/sec. Individual averages varied from 11.5 cm/sec to 19.3 cm/sec. From these data it appears the TPS is non-polarized, although the possibility of normal input polarization is not eliminated by these experiments since electrical stimuli would be expected to activate the conducting system directly. Experiments in which stimulating electrodes were placed at the base and neck, with three recording electrodes between, show very similar-appearing pulses traveling base to neck and neck to base, respectively.

Possible facilitation in the TPS. Some records have been obtained which might be interpreted as showing facilitation (Fig. 4). However, since the recordings were made with suction electrodes it is difficult to evaluate these results because the relation of the electrode to the underlying tissue and the strength of the suction can change over time as the animal expands and contracts.

The distal opening response. Torrey (1904) and Parker (1919) describe the distal opening response in *Corymorpha palma,* and both Wyman (1965) and Krasilovsky (1967, unpublished) apparently had little trouble eliciting it in *Corymorpha palma* and *C. pendula,* respectively. In the present study, on the contrary, consistent distal opening was obtained in only two of more than forty animals tested over the period of approximately a year, with a few other animals giving an inconsistent response. Since a majority of the specimens of *Tubularia*

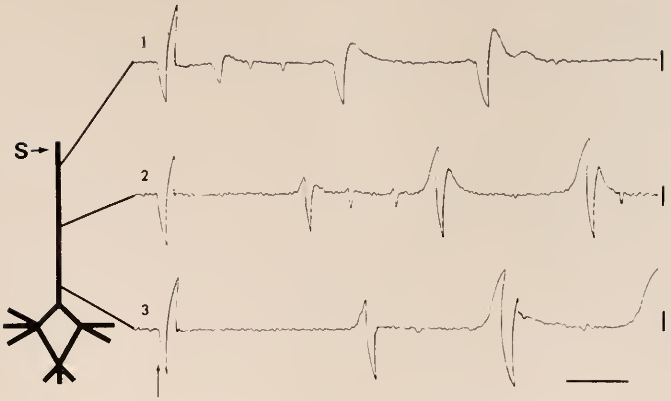


FIGURE 4. Example of possible facilitation during a triggered burst in *Corymorpha*. Vertical scale equals 50 μV; horizontal scale equals 0.1 sec.

tested gave a distal opening response with a similar stimulating set-up, the difficulty encountered in obtaining this response has yet to be explained.

In two specimens of *Corymorpha* distal opening was sometimes obtained without any other observable response. In one case the response was associated with a burst of large pulses; in the other there was a single small pulse (Fig. 5). In those animals where there is a burst of large pulses associated with the distal opening response, it is often impossible to distinguish between bursts which are associated with distal opening and those which are not.

Several lines of evidence point to the possible existence of more than one pulse system. First, there are often two fairly distinct sizes of pulses (Fig. 6A), or pulses of two distinct waveforms (Fig. 6B). Secondly, several records appear to show differing conduction velocities for large and small pulses (Fig. 6 C, D).

Alterations in conduction velocity in response to continued stimultion. Most animals showed only a slight decrease in conduction velocity following long-con-

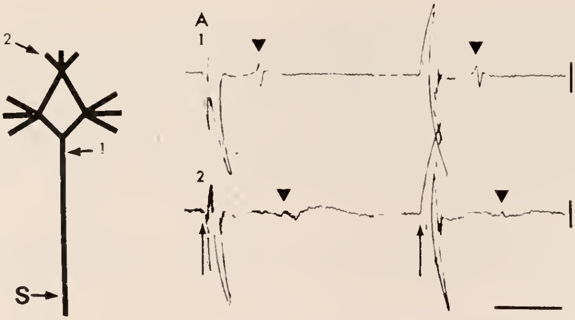


FIGURE 5. The single small pulse (marked by a triangle) associated with distal opening in the absence of any other observable reaction, as recorded from the stalk and distal tentacle of a *Corymorpha*. Portions of the records were retouched for reproduction. Vertical scale equals 200 μV; horizontal scale equals 0.1 sec.

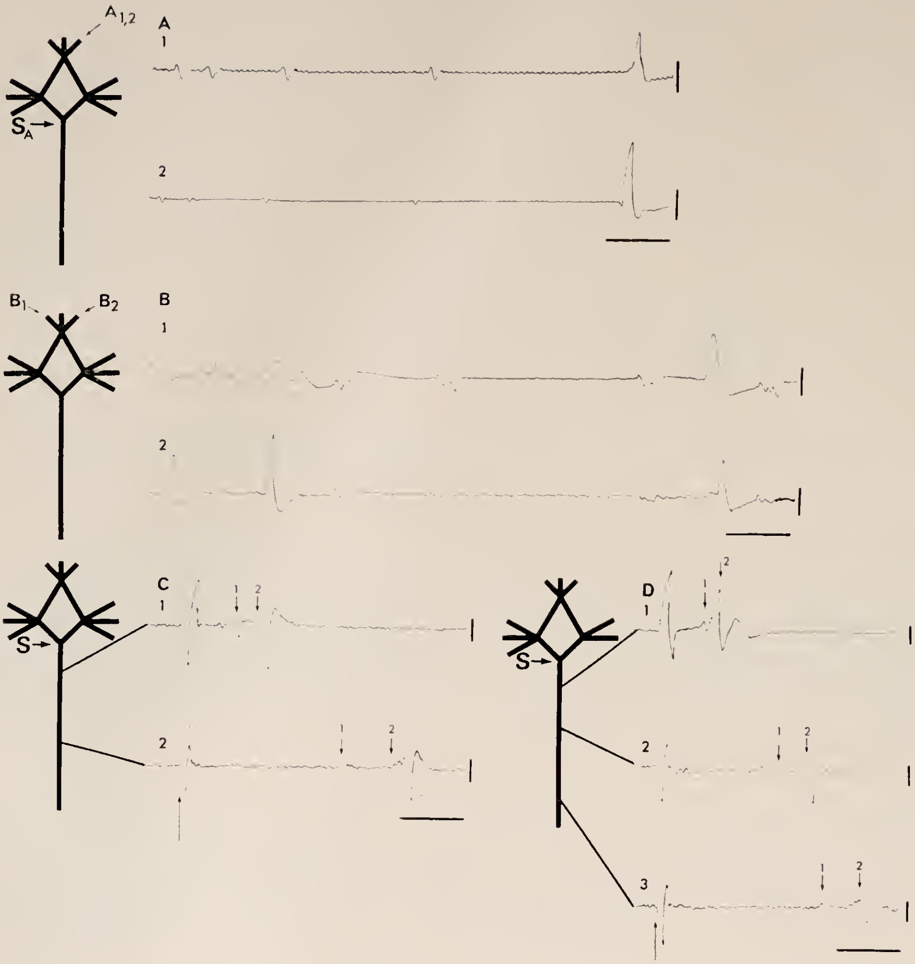


FIGURE 6. Evidence for two conducting systems in *Corymorpha;* (A.) pulses of two distinct sizes; (B.) two types of pulses differing in waveform as well as size. These pulses were triggered, but location of the stimulating electrode was not recorded; (C and D.) evidence for two distinct pulse sizes and conduction velocities in the stalks of two different animals. The two pulses in each record are indicated by numbers and arrows. Conduction velocities of the large and small pulses are, respectively, 13.9 and 17.7 cm/sec in C, and 16.8 and 19.7 cm/sec in D. The vertical scale in A and B equals 200 μV except A2 which is 1 mV, and vertical scale in C and D equals 50 μV; horizontal scale in all records equals 0.1 sec.

tinued stimulation, but in several cases the apparent velocity dropped to about half the original velocity.

Investigations on the layer of origin of triggered stalk pulses

The protocol of the abrasion experiments, which were used to establish the tissue of origin of triggered stalk pulses, can best be discussed together with the

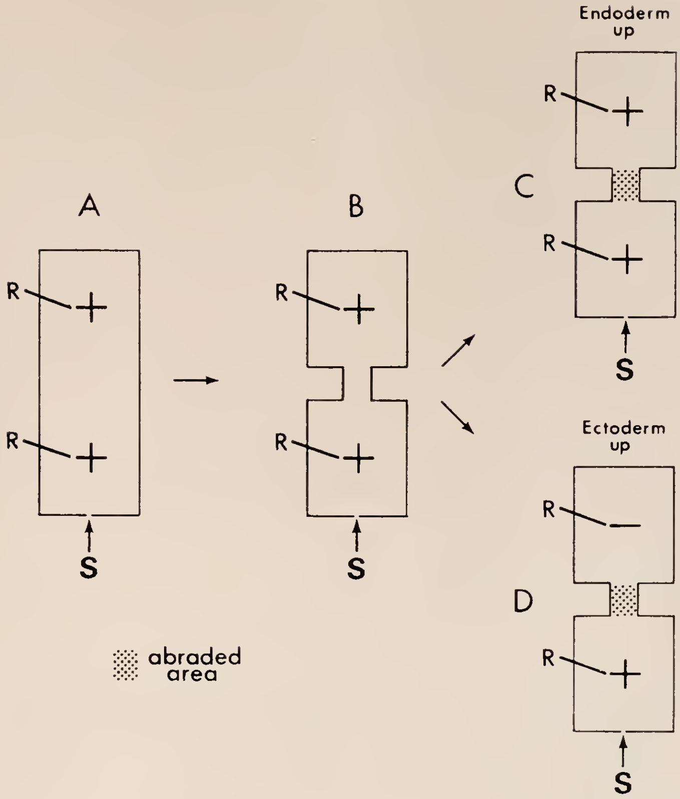


FIGURE 7. Diagrammatic representation of experiment establishing the tissue of origin of triggered stalk pulses in *Corymorpha*. See text for details. S and adjacent arrow indicate the point at which stimuli were applied; R indicates a recording electrode; + indicates the presence of a triggered pulse; — indicates its absence.

results and by reference to Figure 7. A stalk of *Corymorpha* was split longitudinally and pinned out as a sheet of tissue. In three cases this sheet was endoderm up and in three cases ectoderm up. A stimulating electrode was placed at one end and two recording electrodes were placed along what was formerly the long axis of the stalk. Five successive single stimuli were given at 1-minute intervals and either a single pulse or a burst of pulses was recorded on both electrodes in response to each stimulus (Fig. 7A). Transverse incisions were then made in the stalk tissue between the two recording electrodes leaving them connected by a narrow tissue bridge, and five single stimuli were again given at 1-minute intervals with the response being recorded on both electrodes (Fig. 7B). The upper layer of the tissue forming the bridge between the two recording electrodes was then abraded away with a glass needle. The operation could be performed with minimal injury to the underlying tissue because of the remarkably tough mesoglea of *Corymorpha*. When the endodermal side of the bridge was abraded (Fig. 7C)

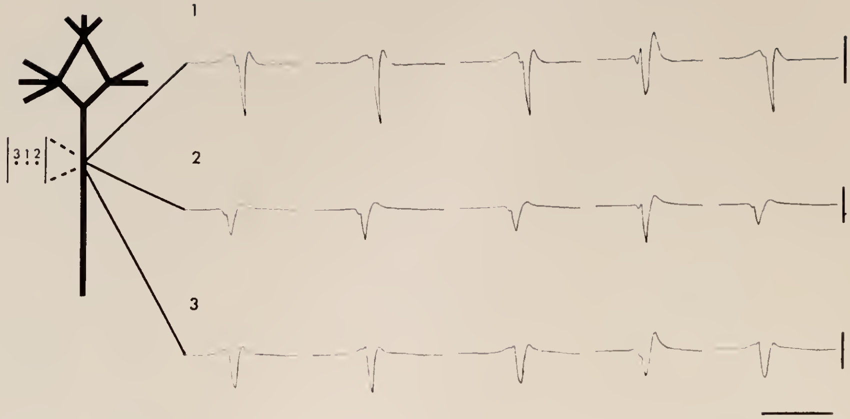


FIGURE 8. Records from three electrodes lined up transversely across the stalk of a *Corymorpha*. Vertical scale equals 500 μV; horizontal scale equals 0.1 sec.

the pulses continued to pass across the bridge, but they failed to do so when the ectoderm was abraded (Fig. 7D).

Investigations of the spatial distribution of the TPS

Localized conduction tracts were sought by recording with three suction electrodes lined up at approximately 1 mm intervals transversely across the stalk of each of two specimens of *Corymorpha*. Records obtained from all of the electrodes were relatively similar in time of arrival, waveform, and amplitude (Fig. 8).

In a further effort to detect signs of preferential conduction an experiment of Parker (1919), which is illustrated in Figure 9, was repeated while recording from three suction electrodes placed at various points on the two arms of the preparation. Recordings were made from 3 replicates of each of the three types of preparation. In all three types typical triggered pulses were recorded from both arms following

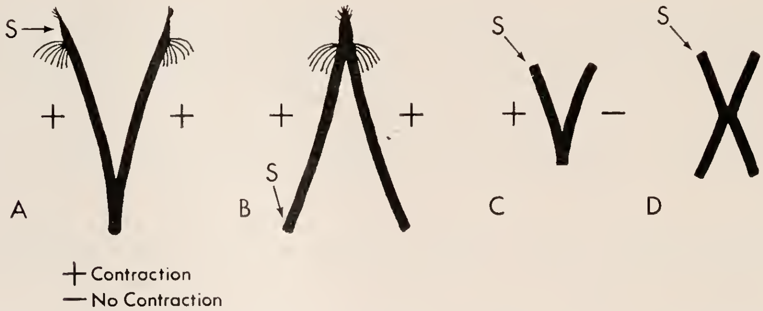


FIGURE 9. Diagrammatic summary of some of Parker's (1919) experiments on conduction pathways in *Corymorpha*. A–C give Parker's results. D is a modification of C used in the present experiments in which the hydranth was removed and the two arms of the preparation remained connected by a bridge of stalk tissue.

Table I

Summary of experiments with drugs and ions

Compound	Concentration	Preparation	Replicates	Effect
				I. *Drugs*
Tyramine HCl	0.01 M	Whole animal	3	General increase in activity as well as an increase in bursting tendency which was especially noticeable in the proboscis
Tyramine HCl	0.01 M	Excised hydranth	3	General increase in electrical activity with an increase in bursting tendency which was especially noticeable in the proboscis (Fig 11).
Tyramine HCl	0.01 M	Excised proximal tentacles	3	General increase in electrical activity (3 PTs) and in bursting tendecy (2 PTs).
D-Amphetamine SO_4	0.01 M	Excised hydranth	3	No clear change in electrical activity.
Ephedrine HCl	0.01 M	Whole animal	6	Pulse size was generally reduced. Some parts of the animal showed increased electrical activity, while that in other parts was reduced. No consistent pattern of change was apparent.
Ephedrine HCl	0.005 M	Whole animal	3	Two animals showed a clear increase in electrical activity; the third showed little change.
Ephedrine HCl	0.01 M	Excised hydranth	4	A fairly consistent pattern with the proximal tentacles showing a burst immediately on application of the drug, then becoming almost totally inactive, while proboscis activity changed almost entirely to regular, rather sharp bursts with correlated concert activity (Fig. 12A).
Ephedrine HCl	0.01 M	Excised proximal tentacles	9	Seven of nine tentacles showed a considerable increase in pulse activity (Fig. 12B).
Phenylpropanolamine	0.01 M	Whole animal	3	Tremendous increase in electrical activity in one animal (Fig. 13A), a slight increase in another, and no apparent change in a third.
Phenylpropanolamine	0.01 M	Excised hydranth	3	Considerable increase in electrical activity with all pulses grouped into bursts (Fig. 13B).
Phenylpropanolamine	0.01 M	Excised proximal tentacles	3	Activity increased to a steady stream of pulses.
Tetrodotoxin	10^{-5} g/ml	Excised hydranth	2	No change in spontaneous electrical activity within 60 minutes in one case or 195 minutes in the other.
Tetrodotoxin	10^{-5} g/ml	Sheet of stalk tissue	1	No effect on either spontaneous or stimulus-induced pulses within 312 minutes.

TABLE I—(*Continued*)

Compound	Concentration	Preparation	Replicates	Effect
				II. *Inorganic ions*
$MgCl_2$	Isotonic (0.54 M)	Whole animal	3	Pulses were eliminated, but were restored when the animal was returned to sea water.
$MgCl_2$	1 part isotonic: 9 parts sea water	Whole animal	3	A definite slowing of electrical activity in two cases; no effect in the other.
Ca^{++}-free Sea Water	Isotonic	Whole animal	3	Pulses became smaller and less frequent. Animals assumed a frozen appearance without showing any obvious relaxation.

stimulation of one. In the preparation with the two arms joined basally (Fig. 9A) the perisarc was slipped off and a recording electrode was placed near the base. There was clear conduction through the basal portion of the stalk as evidenced

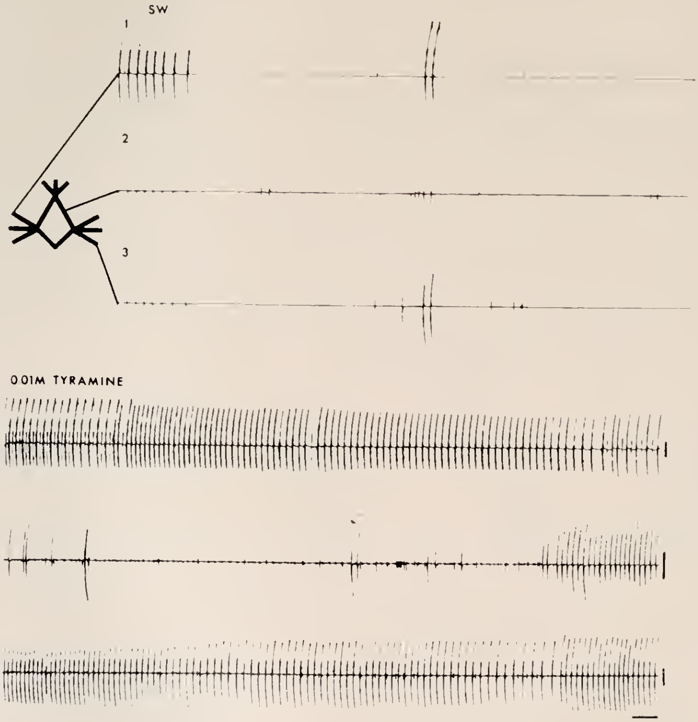


FIGURE 10. Effect of 0.01 M tyramine HCl on electrical activity in an excised hydranth. Record is continuous except that the amplifiers were turned off during the change of solutions. Vertical scale equals 1 mV; horizontal scale equals 5 sec.

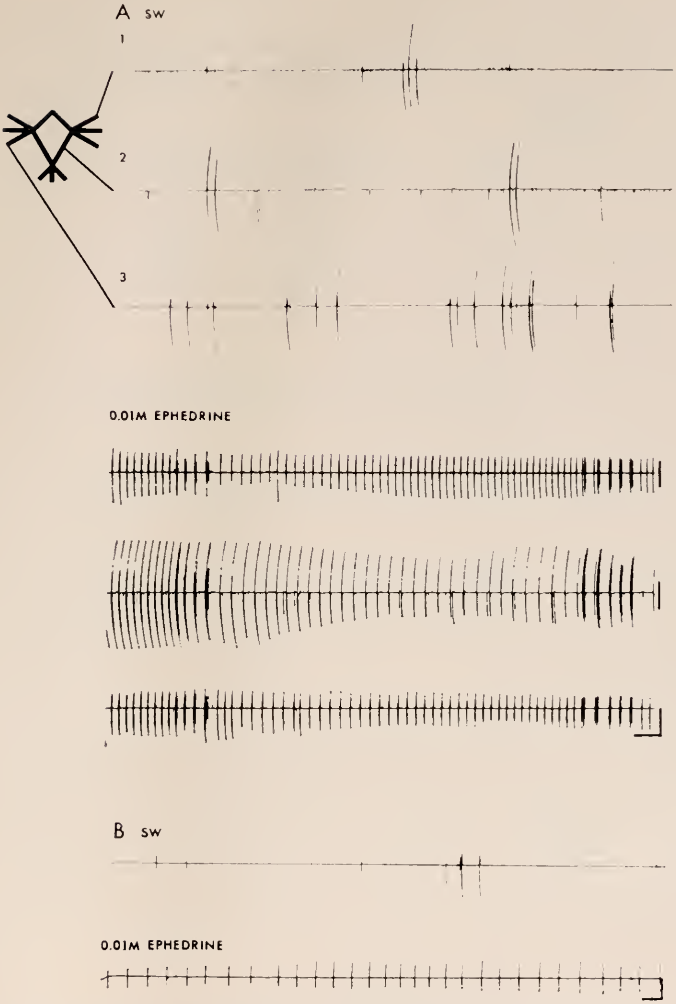


FIGURE 11. Effect of 0.01 M ephedrine HCl on electrical activity of excised parts of *Corymorpha;* (A.) an excised hydranth; (B.) an excised proximal tentacle. Records are continuous except that the amplifiers were turned off during the change of solutions. Vertical scale equals 1 mV; horizontal scale equals 5 sec.

by large pulses in both arms of the preparation; but within the area normally covered with perisarc the pulses were greatly reduced in amplitude.

Experiments involving drugs

Most of the results are summarized in Table I and Figures 10 through 13. Some of the findings presented in Table I are discussed more extensively below along with those results which could not be adequately presented in tabular form.

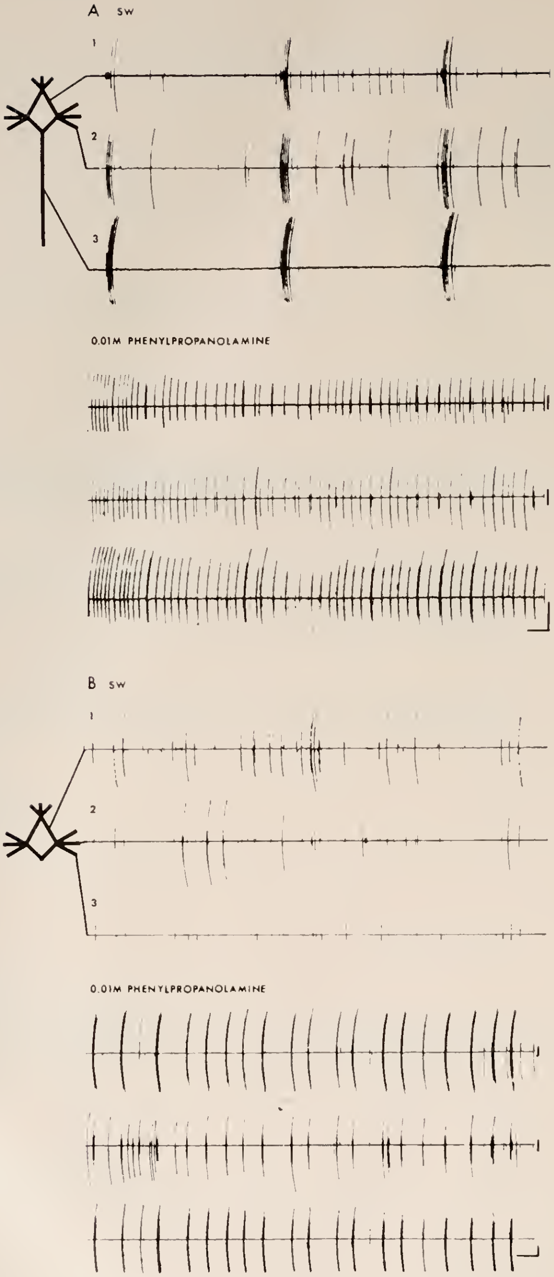


FIGURE 12. Effect of 0.01 M phenylpropanolamine on electrical activity of an intact *Corymorpha* and of excised parts; (A.) an intact animal, record continuous; (B) an excised hydranth. Record shows activity approximately one minute after drug application. Vertical scale equals 500 μV; horizontal scale equals 5 sec.

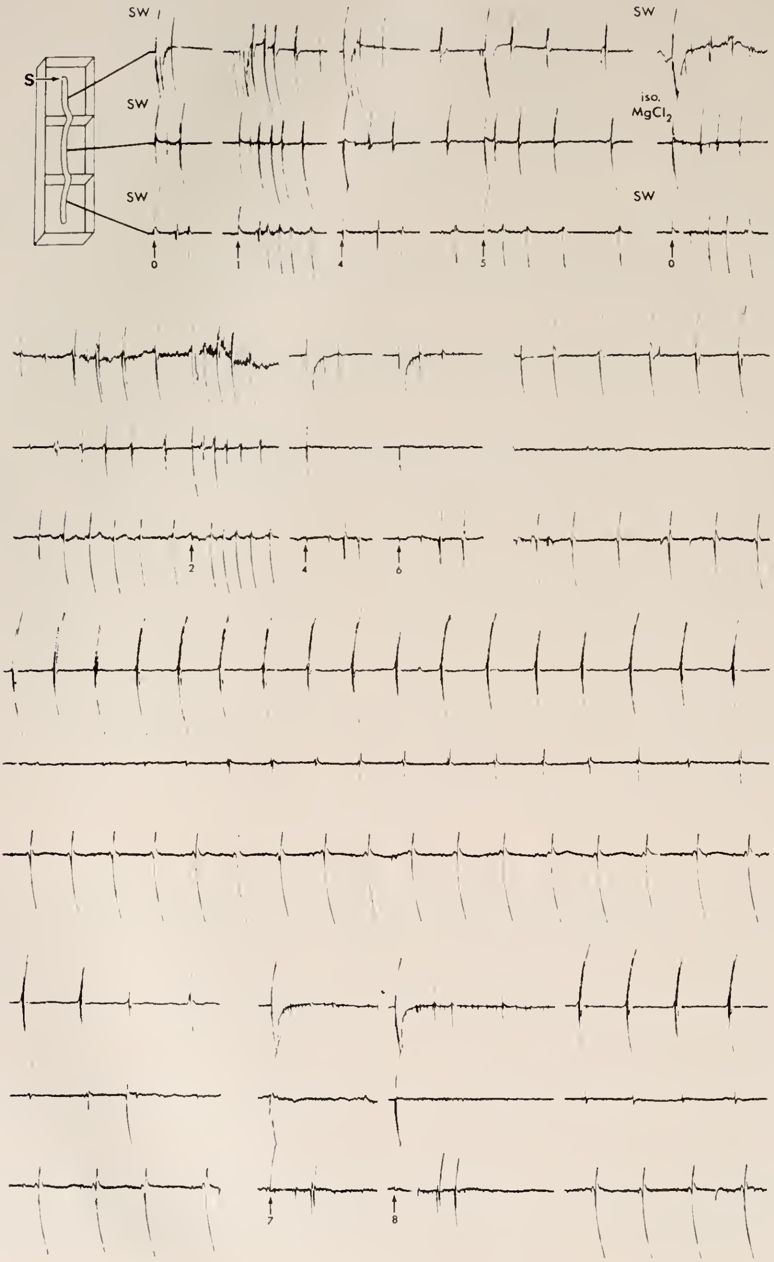
Ca^{++}-free sea water had rapid and clear-cut effects on electrical and muscular activity. Although there was no apparent muscular relaxation almost all motion ceased within 15 minutes in each of three animals which were placed in Ca^{++}-free sea water. Pulse amplitude generally became smaller and pulses less frequent, although they continued long after all motion had disappeared. Following reapplication of sea water the distal tentacles were in motion within 30 seconds even when they had been in Ca^{++}-free sea water for up to 270 minutes. Within 5 minutes the proximal tentacles and proboscis were also back in motion.

All five of the isolated stalks which were run in the three-chamber bath with central $MgCl_2$ substitution gave essentially the same results, although in several instances complete recovery of the large pulses in the middle chamber following return to sea water was not obtained. Electrode slippage sometimes made repositioning necessary. In general, the pattern was that shown in Figure 13, with little change in the pulse pattern in response to stimulation for at least the first 2 minutes after the addition of $MgCl_2$ to the middle chamber. Shortly thereafter the electrical response of the portion of the stalk in the middle chamber began to decrease until it eventually disappeared, as shown in Figure 13 at minutes 4 and 6 after the addition of $MgCl_2$. Conduction persisted through the portion of the stalk in this compartment, however, as evidenced by continuing full-sized pulses in compartments 1 and 3. The record shown in Figure 13 is unusual in that when the recorder was turned on preparatory to giving the seventh stimulus following addition of $MgCl_2$ a spontaneous burst was found to be in progress, as evidenced by the large pulses recorded in chambers 1 and 3. For approximately the first half of the burst there was no response from the portion of the stalk in $MgCl_2$. Then a small pulse began to appear which grew in amplitude with successive pulses of the burst. Once the portion of the stalk in the central chamber had repeatedly failed to respond to electrical shocks (at minutes 7 and 8 after addition of $MgCl_2$ in Figure 13) the $MgCl_2$ in the central chamber was replaced with sea water. Stimulation was then continued at random intervals to check the recovery of large pulses in the central chamber. Recovery usually occurred (minutes 48 and 49 after return to sea water in Figure 13), although in several cases the pulses failed to regain their original amplitude.

Discussion

Several of the differences between the spontaneous pulse systems and conducting systems of *Corymorpha* and *Tubularia* can be attributed to the presence of well-developed epitheliomuscular cells in the stalk of *Corymorpha*. The stalk of *Tubularia*, by comparison, is covered with perisarc and the muscle cells beneath this are apparently greatly reduced or absent (Hyman, 1940). Functionally *Corymorpha* can be regarded as a *Tubularia* with an elongate neck.

The triggered pulses produced in both the stalk of *Corymorpha* and the neck of *Tubularia* appear to be identical to pulses spontaneously originating in these areas. Originally no electrical correlate of the triggering system had been recorded in the stalk of *Tubularia*, but R. K. Josephson (University of California, Irvine, personal communication) has recently recorded small labile pulses associated with this system. This is again similar to the situation in *Corymorpha* where pulses are greatly reduced in amplitude when passing through the perisarc-covered base. On the basis of these findings there appears to be a clear association between the presence of a well-developed musculature and the generation of the large electrical pulses discussed in this and the preceeding paper.



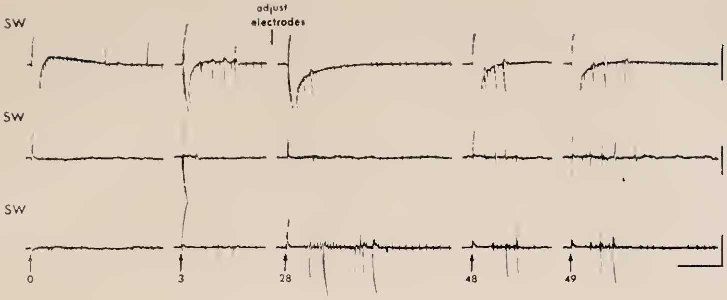


FIGURE 13. Magnesium chloride bridge experiment. Stimulus artifacts are marked by upward-pointing arrows beneath the records. The numbers beneath the arrows indicate the time (minutes) since the start of that particular portion of the experiment. See text for further details.

A distal opening response is present in *Corymorpha,* though judging from the literature this response should have been obtained much more readily and consistently than it actually was. From the electrical records obtained in the present study it is impossible to say that there is a distal opening system which is clearly separable from the TPS. Much time was spent on many animals unsuccessfully trying various methods of eliciting consistent distal opening. Fresh animals were no more responsive or consistent than those which had been in the laboratory for some time.

Nothing comparable to the slow system described by Josephson (1965) in *Tubularia* has been observed.

Neither of the experiments which might have yielded evidence for longitudinal conduction tracts in the stalk (the experiment with three electrodes arranged transversely across the stalk and Parker's experiment shown in Fig. 9) gave such evidence. However, these experiments certainly do not disprove the existence of such tracts since the suction electrodes used were non-focal and the tissue bridges in the repeat of Parker's experiment might have been too wide to reveal preferential conduction.

Kass-Simon (1972) has found that in *Hydra* bridge width is critical in determining whether or not there is differential conduction in the two directions; bridges less than 100 μ or 150 μ and wider showing no significant differences in conducting ability. In view of Parker's evidence for preferential conduction in *Corymorpha* and the fact that the bridges used in our experiments were greater than 150 μ preferential longitudinal conduction in *Corymorpha* must still be considered a strong possibility.

The failure of tetrodotoxin to block electrical activity in both *Corymorpha* and *Cordylophora* (Mackie, 1968) is certainly of interest, but beyond that little can be said in the absence of intracellular recording.

Most of the sympathomimetic drugs which were tested altered the spontaneous electrical activity of *Corymorpha.* Their most consistent effect was to change electrical activity in the proboscis to brief high-frequency bursts occurring at short intervals. In addition, overall electrical activity was increased, especially in tyramine and phenylpropanolamine. Several of the drugs had a more marked effect on isolated parts than they did on the whole animal, but it is not known whether this result has anything to do with functional properties of the system or whether it is merely a product of reduced physical restraint of the isolated parts. Another possibility is that the drugs were able to permeate the tissues more fully through recently cut areas.

In these experiments our goal was to study the relationship between electrical activity and behavior, so we used high drug concentrations in an effort to produce clear changes. Therefore any possible role of sympathomimetic or related compounds as transmitters in *Corymorpha* remains in doubt, In view of the effects of such compounds on *Tubularia* (Parmentier and Case, 1973) and *Corymorpha* an examination of these hydroids using histochemical localization techniques (Dahl, Falck, Von Mecklenberg and Myhrberg, 1963) would be of considerable interest.

Within the limits of individual variability there was generally a change in behavior associated with altered electrical activity. In those excised hydranths which showed major alterations in electrical activity in the presence of drugs, each burst of HPs was associated with at least a partial concert, although this concert was often apparent only as a simultaneous inward twitch of all the proximal tentacles which never relaxed fully between the rapidly repeated bursts. In excised proximal tentacles to which drugs had been applied the relation between electrical activity and movment was much less clear. Sometimes a steady stream of pulses was produced without apparent movement; but even in normal isolated tentacles in sea water an obvious flexion often occurs on only the first pulse of a burst. Due to our failure to obtain a consistent distal opening response from *Corymorpha* we were unable to test the effects of drugs on the operation of this system, as was done by Parmentier and Case (1973) with *Tubularia*.

The blockage of neuromuscular transmission by excess magnesium is well established (Ross and Pantin, 1940; Bullock, 1943; Engbaek, 1952). In both cholinergic (Hubbard, Jones and Landau, 1968) and adrenergic (Kirpekar and Wakade, 1968) systems it has been suggested that the decrease in the amount of transmitter released, which is the most important factor in the blockage of neuromuscular transmission, is due to competition between Mg^{++} and Ca^{++}.

However, excess Mg^{++} apparently also can affect nerve directly. Mackie and Passano (1968), in an extensive discussion of the relation between neural and epithelial activity in hydromedusae, state that excess Mg^{++} rapidly eliminates electrical activity in systems which are known to contain nerves but in which epitheliomuscular cells may also be involved, whereas it does not prevent propagation in purely epithelial conducting systems within a comparable time span, if at all.

Before attempting to put together the results presented in this and the preceding paper into a coherent picture of the relation between electrical activity and behavior in *Corymorpha* it would appear to be worthwhile to briefly summarize the major points which such a model must accommodate. They are: 1. Most spontaneous pulses are associated with obvious contractions of the longitudinal musculature. 2. The characteristics of pulses produced by all of the spontaneous pulse systems overlap, suggesting that all of the pulses may have a common origin in epitheliomuscular cells. 3. Stalk pulses, and by analogy all other pulses associated with contraction of the longitudinal muscles, are conducted on the ectodermal side of the mesoglea. 4. Much of the behavior of *Corymorpha* which does not involve contractions of the ectodermal musculature has no recordable electrical correlate.

5. When alterations in behavior involving the ectodermal musculature are produced there is generally a corresponding change in electrical activity. 6. The processes of electrogenesis of large pulses and conduction can be separated in a bridge of tissue treated with $MgCl_2$. Also, there is conduction through perisarc-covered areas, which have a poorly developed musculature, without production of large pulses.

Points 1–5 could all be explained by hypothesizing that the activity which we have recorded consists of muscle potentials which are a concomitant of behvaior and play no role in its control. However, both the observations of Josephson and Mackie (1965) on *Tubularia* and the present studies on *Corymorpha* indicate that when either the whole animal or an excised hydranth is placed in $MgCl_2$ all visible contraction ceases considerably before any apparent change in electrical activity. Similar results were obtained when *Corymorpha* was placed in Ca^{++}-free sea water. The theories which we present below are inadequate to the extent that they fail to explain these results.

The $MgCl_2$ bridge experiments clearly separate the processes of electrogenesis of large pulses and conduction, but, in view of the apparent multiple effects of excess Mg^{++}, there are still several ways in which the results of these experiments could be interpreted. One possible explanation is that a sheet of epitheliomuscular cells can conduct without producing large pulses, either because the two processes, although occurring in the same cells, are separable, or because only a few of the cells are present (beneath the perisarc) or still capable of conducting (within a Mg^{++} treated bridge). Some form of electrical conduction would appear to be the only means by which an impulse could cross such a wide bridge of tissue at the observed speed, yet no electrical activity is recordable near the middle of the bridge. We doubt whether an electrotonically conducted pulse unrecordable at the center would be able to cross the rest of the bridge with sufficient strength to initiate a new wave of epithelial activity on the other side. A second hypothesis, which appears more likely to us, is that excess Mg^{++} has uncoupled the epitheliomuscular cells from activity in the underlying nerve net, possibly through interference with synaptic transmission between nerve cells and overlying epitheliomuscular cells. Excess Mg^{++} is known to affect neuromuscular junctions, and conduction across the high Mg^{++} area can be explained by hypothesizing that the conducting ability of the nerve continues unimpaired even though its ability to locally trigger the epitheliomuscular cells has been blocked by excess Mg^{++}.

It is obvious that many of the uncertainties in the above discussion could be removed by intracellular recordings from epitheliomuscular cells. However, all of our attempts to obtain such records have been unsuccessful (Ball, 1971), so our conclusions must be drawn from the evidence presented above. On this basis we feel that the large electrical pulses recordable from *Corymorpha* are produced in the epitheliomuscular cells, are locally propagated and provide only an incomplete picture of activity in the nervous system which is actually controlling behavior.

Many of the types of spontaneous electrical activity recorded from other hydroids are associated with obvious muscular activity and in most cases a nerve net is present running among the bases of the epitheliomuscular cells. Hypothesizing conducting epithelia as additional channels of information transfer appeared to make the explanation of the complex behavior of hydroids somewhat easier since it lessened the need for postulating different conduction pathways and effector sensitivities for activity in a single nerve net. From studies on medusae and siphonophores (Mackie and Passano, 1968) it is clear that epithelia can serve to transmit information from one part of an animal to another and it therefore seems likely that such mechanisms should also be present in hydrozoan polyps. However, we feel that the results of our study suggest caution in accepting conducting epithelia as a means of information transfer in hydroid polyps until such time as the situation can be clarified by intracellular recording from epitheliomuscular cells.

It is a humbling fact that although our equipment was more sophisticated and we sometimes doubted his interpretations along the way, the views which we have finally reached concerning the role of the nerve net in controlling the behavior of *Corymorpha* are very similar to those expressed by G. H. Parker in the *Elementary Nervous System* in 1919.

We thank Dr. James Morin and Dr. R. K. Josephson for helpful discussions during both research and writing. The research was supported by an NDEA Title IV Predoctoral Fellowship (E. E. B.) and by PHS Grant NS-08599 to J. F. Case. This paper is based on part of a thesis presented by E. E. B. to the Dept. of Biological Sciences, University of California, Santa Barbara, in partial fulfilment of the requirements for the Ph.D.

SUMMARY

1. The triggered pulses (TPs) most commonly produced by *Corymorpha* in response to electrical stimulation appear similar to spontaneously occurring stalk pulses (SPs) in amplitude, waveform, and duration. Average conduction velocities in the triggered and spontaneous pulse systems are 15.7 cm/sec and 15.9 cm/sec, respectively. The TPS is non-polarized. Some animals show a relatively consistent threshold of activation; in others the threshold varies over time.

2. The behavioral response to electrical activation of the TPS varies from an almost imperceptible contraction to a vigorous inward flexion of the proximal tentacles associated with strong stalk contraction. In general, there is a clear relation between the length of a burst of electrical activity and the strength of response.

3. A small percentage ($<20\%$) of the *Corymorpha* examined showed a distal opening response, in which all distal tentacles simultaneously flared aborally, to electrical stimulation. In a few animals this response occurred in the absence of other behavior and was associated with a single small electrical pulse; in others it was associated with contraction and a burst of TPs. Only two of more than forty animals gave a consistent distal opening response. Available data do not provide an adequate basis for establishing the DOS as a system distinct from the TPS.

4. Evidence for more than one stalk conducting system is provided by the presence of two sizes of pulses which are conducted at different velocities.

5. Experiments involving the selective destruction of endoderm or ectoderm with a glass needle were used to establish that triggered stalk pulses are conducted in the ectoderm.

6. Three electrodes lined up transversely across the stalk of a *Corymorpha* all record rather similar pulses during a spontaneous stalk burst.

7. Electrical recording from various sorts of bridge preparations provides no evidence for preferential longitudinal conduction tracts.

8. The sympathomimetric drugs tyramine, ephedrine, and phenylpropanolamine tend to cause an immediate increase in electrical activity and grouping of hydranth pulses into short high-frequency bursts. Associated with this alteration in electrical activity is an increase in the frequency of concert behavior. Another sympathomimetic drug, D-amphetamine, causes no clear change in electrical activity.

9. Tetrodotoxin (10^{-5} g/ml) has no effect on either spontaneous or triggered pulses for periods of up to 312 minutes.

10. Ca^{++}-free sea water causes whole *Corymorpha* to become motionless without apparent relaxation of the muscles. Accompanying this effect is a decrease in pulse amplitude and frequency, although pulses continue long after the animal is motionless. On return to sea water the distal tentacles are in motion within 30 seconds, followed by the proximal tentacles and proboscis within a few minutes. Electrical pulses quickly regain their full amplitude and pulse frequency begins to rise.

11. $MgCl_2$ has various effects depending on the concentration and length of exposure. The first effect is to eliminate obvious muscular activity while leaving electrical activity apparently unchanged. A few minutes later electrical activity is also reversibly eliminated. Exposure of the central portion of an isolated stalk to isotonic $MgCl_2$ while the two ends remained in sea water results in the reversible elimination of large pulses in the portion of the stalk exposed to $MgCl_2$ without interfering with conduction through the area or with the generation of large pulses at either end of the stalk. This result is interpreted as indicating that the epitheliomuscular cells are locally triggered to generate large electrical pulses by activity in the nerve net.

LITERATURE CITED

Ball, E. E., 1971. Electrical activity and behavior in the solitary hydroid, *Corymorpha palma* Torrey. *Ph.D. thesis, University of California, Santa Barbara.*

Bullock, T. H., 1943. Neuromuscular facilitation in scyphomedusae. *J. Cell. Comp. Physiol.*, **22**: 251–272.

Cavanaugh, G. M., Ed., 1956. *Formulae and Methods V. of the Marine Biological Laboratory Chemical Room.* Marine Biological Laboratory, Woods Hole, Massachusetts, 87 pp.

Dahl, E., B. Falck, C. von Mecklenberg and H. Myhrberg, 1963. An adrenergic nervous system in sea anemones. *Quart. J. Microscop. Sci.*, **104**: 531–534.

Engbaer, L., 1952. The pharamcological actions of magnesium ions with particular reference to the neuromuscular and cardiovascular system. *Pharmacol. Rev.*, **4**: 396–414.

Hubbard, J. I., S. F. Jones and E. M. Landau, 1968. On the mechanism by which calcium and magnesium affect the release of transmitter by nerve impulses. *J. Physiol.*, **196**: 75–86.

Hyman, L. H., 1940. *The Invertebrates: Protozoa through Ctenophora.* McGraw-Hill, New York, 726 pp.

Josephson, R. K., 1961. Colonial responses of hydroid polyps. *J. Exp. Biol.*, **38**: 559–577.

Josephson, R. K., 1965. Three parallel conducting systems in the stalk of a hydroid. *J. Exp. Biol.*, **42**: 139–152.

Josephson, R. K., 1967. Conduction and contraction in the column of *Hydra. J. Exp. Biol.*, **47**: 179–190.

JOSEPHSON, R. K., AND G. O. MACKIE, 1965. Multiple pacemakers and the behaviour of the hydroid *Tubularia*. *J. Exp. Biol.*, **43**: 293–332.

JOSEPHSON, R. K., AND M. MACKLIN, 1967. Transepithelial potentials in *Hydra*. *Science*, **156**: 1629–1631.

JOSEPHSON, R. K., AND M. MACKLIN, 1969. Electrical properties of the body wall of *Hydra*. *J. Gen. Physiol.*, **53**: 638–665.

JOSEPHSON, R. K., AND J. UHRICH, 1969. Inhibition of pacemaker systems in the hydroid *Tubularia*. *J. Exp. Biol.*, **50**: 1–14.

KASS-SIMON, G., 1972. Longitudinal conduction of contraction burst pulses from hypostomal excitation loci in *Hydra attentuata*. *J. Comp. Physiol.*, **80**: 29–49.

KIRPEKAR, S. M., AND A. R. WAKADE, 1968. Release of noradrenaline from the cat spleen by potassium. *J. Physiol.*, **194**: 595–608.

KRASILOVSKY, G. H., 1967. A contribution to the understanding of the behavior of *Corymorpha pendula*. [Unpublished Post-Course Project Report, Invertebrate Zoology Course, Marine Biological Laboratory, Woods Hole, Massachusetts.]

MACKIE, G. O., 1968. Electrical activity in the hydroid *Cordylophora*. *J. Exp. Biol.*, **49**: 387–400.

MACKIE, G. O., AND L. M. PASSANO, 1968. Epithelial conduction in hydromedusae. *J. Gen. Physiol.*, **52**: 600–621.

MORIN, J. G., AND I. M. COOKE, 1971. Behavioural physiology of the colonial hydroid *Obelia*. II. Stimulus-initiated electrical activity and bioluminescence. *J. Exp. Biol.*, **54**: 707–721.

NAKAMURA, Y., S. NAKAJIMA AND H. GRUNDFEST, 1965. The action of tetrodotoxin on electrogenic components of squid giant axons. *J. Gen. Physiol.*, **48**: 985–996.

NARAHASHI, T., J. W. MOORE AND W. R. SCOTT, 1964. Tetrodotoxin blockage of sodium conductance increase in lobster giant axons. *J. Gen. Physiol.*, **47**: 965–974.

PARKER, G. H., 1917. The activities of *Corymorpha*. *J. Exp. Zool.*, **24**: 303–331.

PARKER, G. H., 1919. *The Elementary Nervous System*. J. B. Lippincott, Philadelphia, 229 pp.

PARMENTIER, J., AND J. CASE, 1973. Pharmacological studies of coupling between electrical activity and behavior in the hydroid *Tubularia crocea* (Agassiz). *Comp. Gen. Pharmacol.*, **4**: 11–17.

ROSS, D. M., 1960a. The effect of ions and drugs on neuromuscular preparations of sea anemones. I. On preparations of the column of *Calliactis* and *Metridium*. *J. Exp. Biol.*, **37**: 732–752.

ROSS, D. M., 1960b. The effects of ions and drugs on neuromuscular preparations of sea anemones. II. On sphincter preparations of *Calliactis* and *Metridium*. *J. Exp. Biol.*, **37**: 753–774.

ROSS, D. M., AND C. F. A. PANTIN, 1940. Factors influencing facilitation in *Actinozoa*: the action of certain ions. *J. Exp. Biol.*, **17**: 61–73.

TORREY, H. B., 1904. Biological studies on *Corymorpha*. I. *C. palma* and environment. *J. Exp. Zool.*, **1**: 395–422.

WYMAN, R., 1965. Notes on the behavior of the hydroid, *Corymorpha palma*. *Amer. Zool.*, **5**: 491–498.

Reference: *Biol. Bull.*, **145**: 265–278. (October, 1973)

LUNAR-CORRELATED VARIATIONS IN WATER UPTAKE BY BEAN SEEDS[1]

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Water uptake in pinto beans, *Phaseolus vulgaris*, was chosen as a simple process by which to assay responses to natural atmospheric and laboratory related variations in extremely weak electromagnetic fields.

A previous study (Brown and Chow, 1973) had disclosed that the mean water uptakes of two presumably independent groups of 20-bean samples at separated sites in the laboratory could display day-to-day variations of an extent and character unaccountable in terms of any known environmental variations while exhibiting a coefficient of correlation between them greater than + 0.8. Correlation between two closely adjacent bean samples could be influenced by very slow rotation, 6 rpm, of the pair with the influence differing between clockwise and counterclockwise rotation. Two populations of 20-bean samples simultaneously investigated at two different laboratory sites could, under some special conditions, exhibit between them a strong *negative* correlation in their day-to-day varying water uptake rates. It was postulated (Brown and Chow, 1973) that the beans in their water uptake were able to correlate either positively or negatively with variations in whatever was one of the chief subtle environmental parameters effecting the day-to-day fluctuations, and that other subtle environmental influences could bias the determination of sign.

In the course of further search for characteristics of the fluctuations in the subtle environmental parameters influencing water uptake and apparent interactions among adjacent experimental samples, it was discovered that the water uptake in beans was apparently exhibiting a mean synodic monthly variation. This was noted for a number of experimental series which were initially designed to learn the effects of different numbers and spatial configurations in clusters of vessels, containing beans, upon mutual interactions among them, as well as to extend our knowledge of characteristics of influences of clockwise and counterclockwise rotations and of unstable horizontal magnetic vectors upon interactions among closely adjacent groups of beans.

MATERIALS AND METHODS

Several hundred-pound bags of beans were obtained and stored in the laboratory which was air-conditioned to relatively uniform temperature throughout the year. For the observations reported here the beans were taken from the bags without selection, other than discarding the occasional damaged ones. Twenty beans were placed in a single layer in 6 × 6 cm shallow aluminum screen baskets. The

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[1] This research was supported by N.S.F. grant #GB-31040.

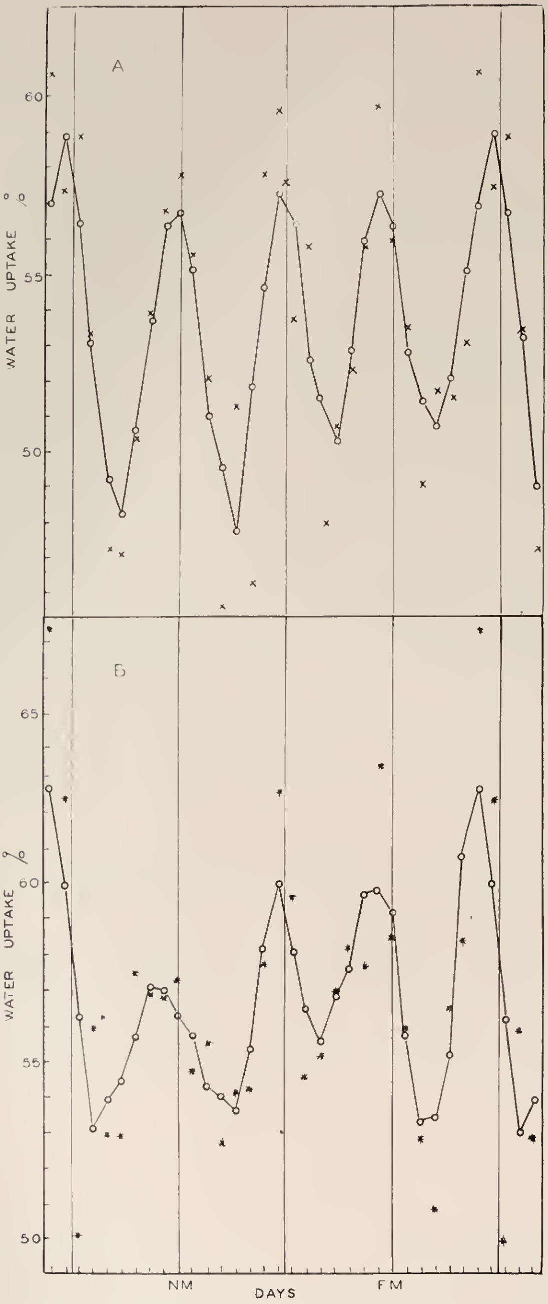

FIGURE 1(A). Mean synodic monthly variational pattern of 4-hour water uptake in Series A (June 27 through November 13, 1972), lines connect 3-day moving mean values; x's indicate individual-day points; (B) mean synodic monthly pattern of 4-hour water uptake in Series B over the same period of Figure 1(A). Symbols are as in Figure 1(A) except that asterisks replace x's.

TABLE 1

Series	Number of 20-bean groups	Calendar period	Days assayed	Results: figures	Mean range
Evanston, Illinois:					
A	12	June 27, 1972–Nov. 13, 1972	86	1A	16.8%
B	9	June 27, 1972–Nov. 13, 1972	91	1B	10.5%
C	16	May 15, 1972–Aug. 18, 1972	66	2A	11.8%
D	12	Sept. 25, 1972–Jan. 22, 1973	70	2B	10.7%
E	12	Sept. 25, 1972–Jan. 5, 1973	65	2C	13.5%
Woods Hole, Massachusetts:					
F	74	June 19, 1972–Aug. 22, 1972	46	3A	12.2%
Evanston, Illinois, Constant-temperature chambers:					
G	4	June 26, 1972–Aug. 24, 1972	41	3B	
H	4	July 26, 1972–Aug. 24, 1972	22	—	
I	4	July 17, 1972–Aug. 24, 1972	28	—	

dry weight of the 20-bean samples ranged typically between 7 and 9 grams. Usually 5 days a week baskets of beans were submerged in tap-water that had been allowed to stand in open containers at room temperature for 12 to 24 hours. One basket was submerged in water in each of 9 × 4.5 cm clear plastic cylindrical vessels. The basket of beans was removed immediately after submergence, pressed onto absorbent tissue, very rapidly weighed to nearest centigram on a torsion balance and resubmerged. After exactly four hours, always spanning the noon hour, the basket of beans was again subjected to same blotting and rapid weighing process. The difference between initial and final wet weights was taken to be the water absorbed. The weight of the absorbed water was expressed as a percentage of the initial dry weight and termed water uptake percentage.

EXPERIMENTS AND RESULTS

Synodic monthly variation in rate of water uptake

The existence of a lunar periodism in rate of water uptake was first noted during analysis of data obtained from two lines of six vessels of beans on a high wooden table in the center of a large laboratory room. The vessels were arranged to form a V with its two arms extending South and East. For this experimental series, A, assays had been made on 86 days distributed over an interval of 140 days (June 27 through Nov. 13, 1972). Correlation between the variations in daily means for the two 6-vessel groups was high, r = +0.83. When the 86 mean daily values for all 12 samples were examined in relation to elongation of moon (new moon ±7 days and full moon ±7 days), a quarter lunar variation was clearly apparent (Fig. 1A). Even for the 3-day moving means which were em-

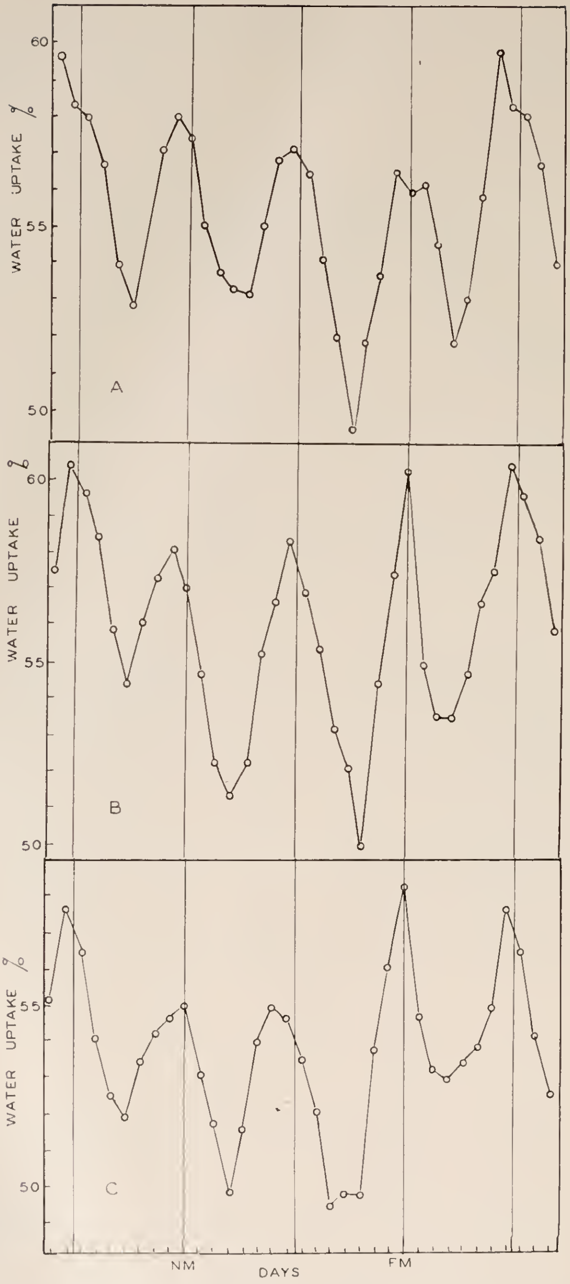

Figure 2(A). Mean synodic monthly pattern for Series C over period May 15 through August 18, 1972, (B) for Series D over period September 25, 1972 through January 22, 1973, and (C) for Series E for the period September 25, 1972 through January 5, 1973. All are three-day moving means.

ployed in recognization of the only approximate relationship between calendar day and moon phase and the many 2-day breaks in daily continuity of assays, the ranges of the mean cyclic variations were observed to be substantial, averaging 16.8% in increases from minima to maxima.

The data for beans in eight other experimental series were then examined. In Table I, these are described with regard to their number of 20-bean samples, span of calendar dates, number of days assayed, figure illustrating results, and mean percentage range of the quarterly cycles. Series A through E were on wooden tables in the open laboratory in Evanston, Illinois; Series F occupied wooden tables at the Marine Biological Laboratory, Woods Hole, Massachusetts, and Series G through I were in three different walk-in constant temperature chambers in Evanston. Despite use of beans from the same laboratory stock supply used in Evanston, the water-uptake rate in the Woods Hole laboratory was substantially higher and no reason was evident.

Series B comprised three triplets of linearly juxtaposed vessels with the three parallelling triplets 55 cm apart. Series D constituted three fully comparably arranged quadruplets. Series E involved six pairs of vessels equally spaced about the periphery of a clockwise rotating (2 rpm) 48-inch circular platform. Series C was one with 8 pairs of 20-bean samples. Two pairs were 50 cm from a slowly rotating (2 rpm clockwise from above) horizontal bar magnet which effected at the location of the beans a ±80° oscillation in a compass needle. Two more pairs, located 17 cm from the magnet were subjected to a periodic 360° rotation of the field at a horizontal vector strength substantially above the earth's natural one. Four other pairs at another laboratory location possessed exactly the same spatial arrangement but no magnet was present. The quarterly monthly variation appeared quite similar in both presence and absence of the rotating magnet and therefore the data for the 8 pairs were pooled.

Series F comprised the pooled results of a number of series including several geometric arrangements, speeds of slow clockwise and counter-clockwise rotation, and the weak field of a rotating magnet.

That conscious bias for lunar periodicity could not have contributed to the results was assured by two factors, (1) more than a dozen different laboratory assistants were involved in data gathering over the several series and (2), the experimental series were already completed, with other objectives in view, before the lunar relationship was discovered.

This demonstration by Figures 1A through 3A of a quarterly (average 7.4-day) lunar variation in bean water uptake, remaining phase-related to the moon's four quarters while encompassing a calendar span of 253 days and entailing 7931 20-bean measurements, leaves no reasonable doubt of a synodic monthly variation in water-uptake by the beans when investigated under these laboratory conditions. The precision in the continuing essentially same relationship to moon phase of the peaks and troughs in water uptake from beginning to end of the more than 8-month span clearly establishes the 7.4-day mean interval between the quarterly peaks as being distinct from any 7-day artifact. Even over the course of four of the presently described six series the quarterly lunar cycles essentially scanned completely the weekly periods. This relative lunar-phase stability of the mean periodism is especially remarkable in the face of a confounding contribution from interaction

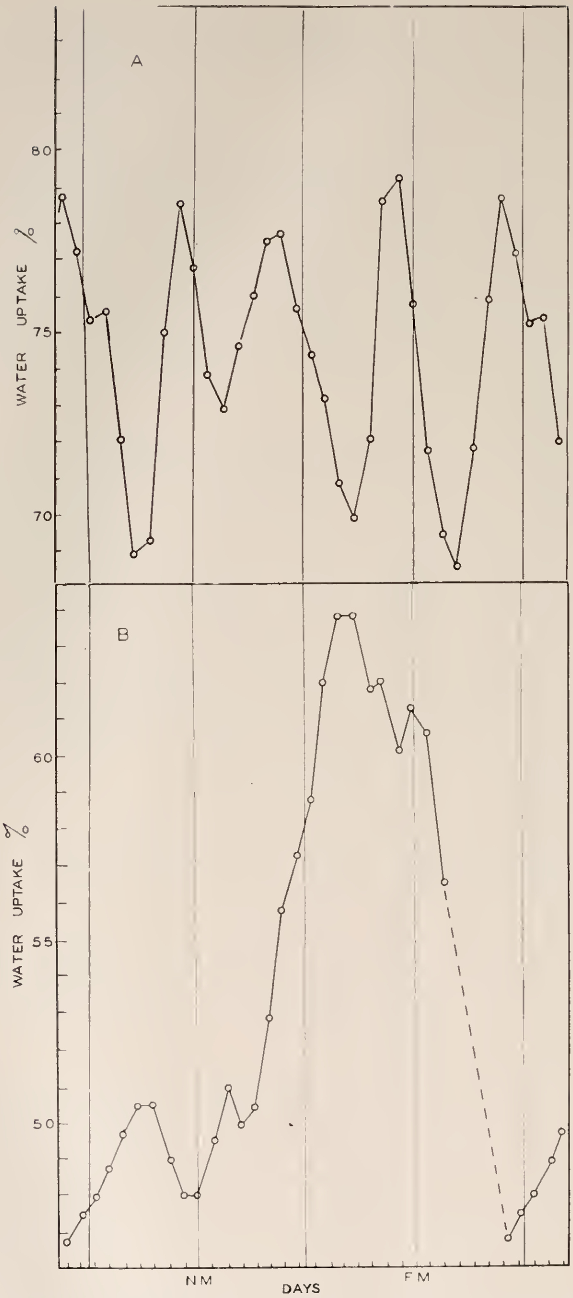

FIGURE 3(A). Mean synodic monthly pattern for bean water uptake for Series F during the period June 19 through August 22, 1972 at Woods Hole, Massachusetts, and (B) Series G in a constant-temperature chamber in Evanston, Illinois, for period June 26 through August 24, 1972. Both are three-day moving means. Broken line indicates gap in data.

between the 5-day-week assays and an apparent systematic semi-annual (minima, spring and fall) fluctuation occurring simultaneously in bean water uptake and emphasizes its polyphasal synodic monthly nature.

No suggestion is present that the period is an autonomous endogenous one for the beans, nor would such be expected in a biological system presumably as biochemically inactive (Bryant, 1972) as a dried bean seed assayed only during its first four hours in water. Instead, these mean cycles give every indication of being simply responses of the beans to lunar-controlled variations in some extremely weak and pervasive environmental parameters. Hence for the purpose at hand use of statistical procedures to determine detailed frequency of the observed variations seems superfluous. Far more valuable and potentially useful and predictable can be the demonstration of a moon-phase relationship.

When a lunar relationship was next sought for some bean series investigated under another, special laboratory condition, namely inside a temperature-controlled chamber, a striking difference was discovered. The earlier study (Brown and Chow, 1973) had described a strong negative correlation between simultaneous variation in water uptake inside (Series G) and just outside the chamber during the interval June 22 through August 4, 1972. Now, it was noted that under such conditions the quarterly lunar cycles also had been induced to exhibit a very conspicuous 180° phase shift.

Not only did minima instead of maxima occur at third quarter and new moon, but an epsecially high and broad maximum occurred between first quarter and full moon (Fig. 3B). Parallel but briefer studies (Series H and I) being conducted in two other constant-temperature chambers of identical type at other laboratory locations disclosed the same peculiar pattern having minima at third quarter and new moon and a very high maximum four days before full moon. Increases from the new moon mimimum to the high maximum for the three chambers were 33%, 41%, and 22%. For all three the duration of the rise and fall in rates in relation to this major maximum encompassed the days of first quarter and full moon.

Over the same calendar period of the foregoing odd patterns within the chambers, and using beans from the same laboratory stock, Series A in Evanston and Series F in Massachusetts were continuing to exhibit their typical quarterly variation. Series B, on the other hand, displayed for this period an inversion of its pattern over the time of new moon with a maximum 3 to 4 days before new moon and a minimum on the day of new moon; the remainder of the monthly pattern possessed its typical quarterly form. This inversion over new moon for this 64-day span within the total of 140 days accounted for the low range of the variation over new moon in Figure 1B.

Synodic monthly variation in interaction among bean samples

An effort was next made to learn whether or not the previously reported apparent interactions between bean samples close together in separate vessels also possibly possessed a lunar variation. Earlier evidence (Brown and Chow, 1973) had suggested that under some circumstances beans in closely apposed vessels mutually induced one another to adopt opposite signs of correlation with subtle geophysical variations, and under other circumstances the same sign. To learn how such alterable interactions might be fluctuating from one day to the next, possibly

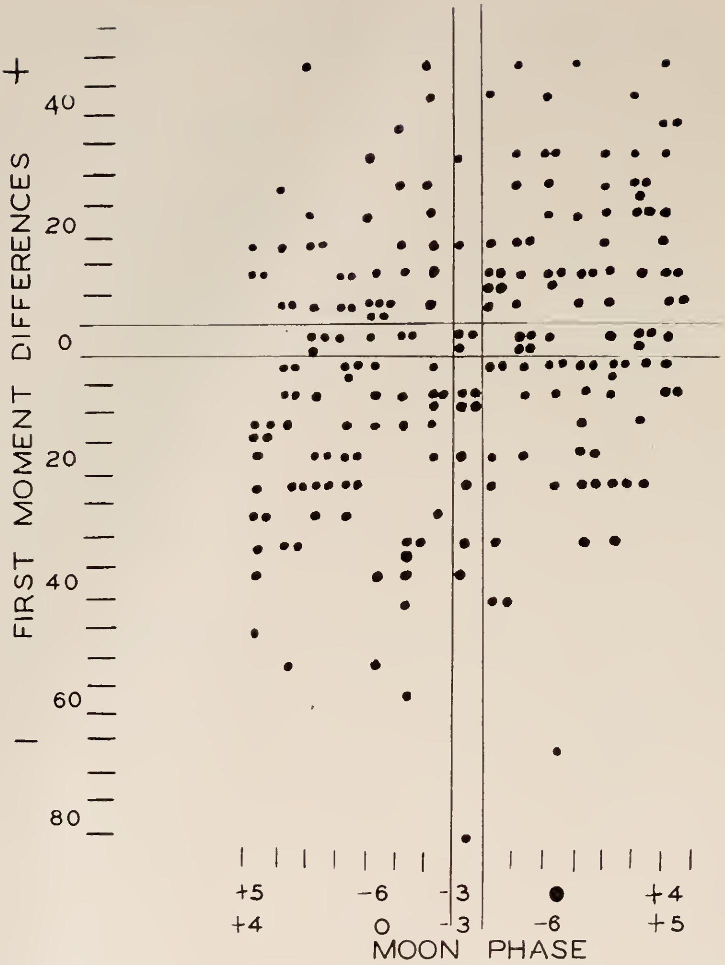

FIGURE 4. Scatterplot of the relationship between moon phase expressed as deviations in days from new moon and full moon and differences between first moment products of closely adjacent bean samples and comparable products of more remotely spaced samples. Included are the pooled results from four extensive experimental series (see text).

even involving a periodic lunar influence, the day-to-day first moments of deviations from their means for each of the several samples in each series were examined. Compared were the first moments, or products of the deviations, of adjacent pairs ("experimentals") and comparable first moments of an equal number of paired samples for which the two vessels were separated usually by 50 cm or more ("controls"). The day-to-day mean differences between first-moment values obtained for closely adjacent vessels and more remotely separated ones were determined. For this analysis, the data for "pairs" *vs* "singles" series previously described (Brown and Chow, 1973), the triplet series, the quadruplet series, and the V-one were used. For the latter three series first moments for alternate vessels in

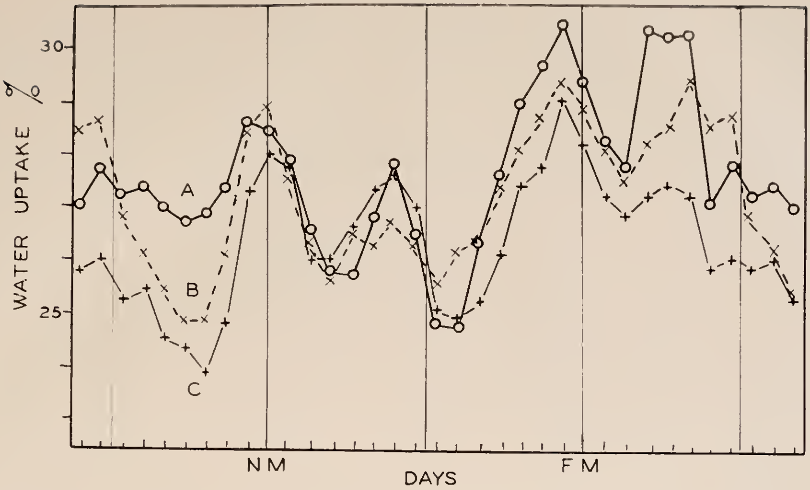

FIGURE 5. Synodic monthly patterns of variation in water uptake of beans over the period Jan. 23 through May 11, 1973 for three concurrent but independent experimental series; A., triplet series; B. quadruplet series; C., rotating magnet series.

a juxtaposed linear series were also determined and included in the pooled results since both the immediately adjacent and alternate vessels appeared to be within the interactional range.

For the "pairs" *vs.* "singles" data, "experimentals" were the three pairs and the "controls" comprised the six "singles." For the triplets and quadruplets the "controls" were the first moments between corresponding members in different ones of the three groups. For the V-series the "controls" were the first moments between members of the opposite groups of six, in reversed sequence.

Inspection of the day to day variation in first moment differences between the "experimentals" and "controls" in the same series suggested occurrence of a monthly variational behavior between vessels located close to one another. A maximum in a *negative* correlation between beans in vessels close to one another relative to concurrent "controls" appeared to occur 4 to 5 days following *new* moon and a maximum in *positive* correlation 4 to 5 days after *full* moon.

Figure 4 is a scatterplot of the relationship of the first moments of "experimentals" minus first moments of "controls" relative to moon phase. These include the mean values for each day of the synodic month for each the adjacents and alternates for all 4 series separately. The scatterplot appears to confirm that the dominant relationship involves greatest positive correlation 4 to 5 days after new moon and greatest negative 4 to 5 days after full moon. There is also suggested within the scatterplot the existence of a lesser inverted monthly relationship. Treating all the data for degree of linear correlation between moon phase and the first-moment differences, r = +0.29; N = 210; t = 4.4.

Experimental alteration of monthly patterns of variation

Additional explorations of the monthly water-uptake variations were made. Beginning January 23, 1973, pinto beans from a new 500-lb lot were used in several experimental series which terminated on May 11, 1973. The rate of water uptake for these beans was much lower despite continuation of the same environmental conditions as for the experiments described earlier. Using these new beans experimental series were carried out in an attempt to confirm the earlier results and to extend knowledge of properties of the phenomenon.

The triplet and quadruplet series were continued in exactly the same manner and places as for the earlier studies. An altered rotating magnet series was investigated. This latter comprised four pairs of vessels around the periphery of a circular table in the center of which a rotating horizontal bar magnet (2 rpm clockwise from above) was present on alternate days. The intervening days served as "controls" for possible influence of the experimental field. The strength of the imposed magnetic field was such as to effect about a ±80° oscillation in a compass needle at the sites of the beans.

In two other series, four pairs of vessels were equidistantly spaced around the edge of circular wooden tables 4 ft in diameter and rotating 2 rpm, one clockwise and the other counterclockwise.

A final two series involved four pairs of vessels (one member of a pair on top of the other) equidistantly spaced around the edge of rotating ($\frac{1}{3}$ rpm), 36-inch circular tables; one table rotated CW and the other CCW. These two series were carried out in a walk-in constant temperature room at 21.5° C, *i.e.*, about 1° lower than for the beans in the open laboratory.

Figure 5 describes the mean synodic monthly patterns of variation for the triplets, quadruplets, and magnetic-field series. For all three there was present a quarterly lunar variation of closely the same percentage range as described for the earlier studies. One difference, however, common to all three series, was an apparent few-day inversion of the quarterly pattern following the time of full moon.

For the series on the 2 rpm rotating tables being investigated in the same large laboratory room and indeed at a site about midway between the forementioned quadruplet and magnet series. The monthly patterns were not only quite evidently different (Figs. 6C and D) from the preceeding three, but differed slightly also between the clockwise (Fig. 6C) and counterclockwise (Fig. 6D) rotations. There is a suggestion that except for a high post-full moon maximum in common the two directional rotations tend to result in generally opposite phase relations.

The two series on the rotating platforms in the constant-temperaure chamber also appeared to display a very slight mirror-imaging tendency between CW (Fig. 6A) and CCW (Fig. 6B) rotations. However, there is a strong suggestion that for a given directional rotation, the beans within the constant-temperature chamber tend to mirror-image the comparable monthly pattern for the beans in the open laboratory outside the chamber. Compare Figures 6A and C, and 6B and D. This provides some confirmation for the odd monthly variations noted the previous summer within these constant-temperature chambers and described earlier.

Also noteworthy is that the water-uptake rate for all four rotating tables reaches essentially the same absolute rate when in apparently the same common phase relationship three to four days following full moon. When adopting a mirror-imaging relationship during most of the remainder of the month, however, the water-uptake rates tend to diverge. Particularly striking is the higher rate of water uptake inside the chamber despite the slightly lower temperature inside the tem-

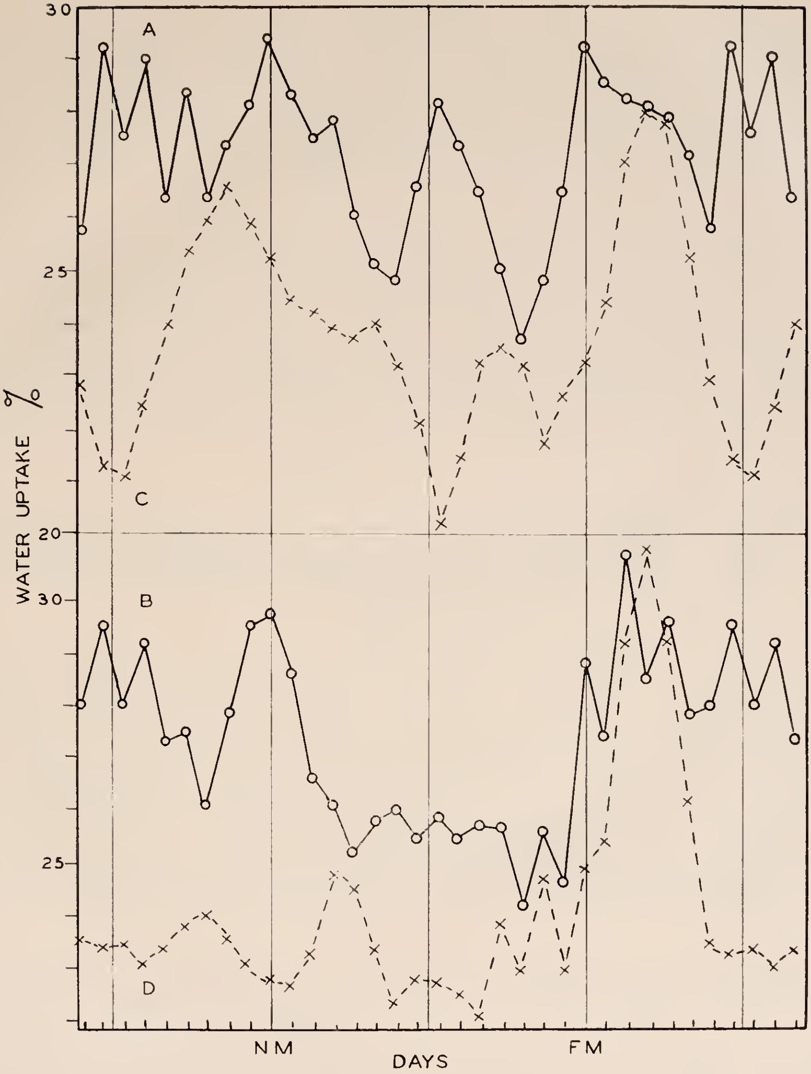

FIGURE 6(A and C). Mean monthly variation in water-uptake rates in beans on uniformly rotating platforms, clockwise from above: (A) at ⅓ rpm inside a walk-in constant-temperature chamber at 21.5° C, and (C) in the general laboratory at 2 rpm and 22.5° C; (B) and (D) with fully parallel conditions to (A) and (C) except that the direction of platform rotation was counter-clockwise.

perature-controlled chamber than outside. This suggests that although the mean rate of water uptake is known to vary with temperature, for any given temperature the rate may differ between whether the sign of correlation with some biologically effective, pervasive, geophysical parameter or parameters is + or −.

DISCUSSION

It seems probable that the effects reported here have been residual ones, algebraic summation of unbalanced positive and negative correlating relationships to the operating external parameters, and that the day-by-day effects themselves can be substantially greater. In fact, there has even been a great reduction in their real scope by the employment of the 3-day moving means (note the x's and asterisks in Fig. 1).

There were no differences in any obvious environmental factors responsible for the odd synodic monthly patterns within the temperature-controlled chambers. One is compelled to conclude, therefore, that the exact manner in which a biological system responds to a monthly variation in some subtle parameters of the atmosphere can be altered by other concurrent, ambient subtle-field states. Such an alteration can apparently be experimentally effected by such laboratory equipment as the temperature-controlled chambers and to a lesser extent by platform rotation. There is thus indication that some additional very weak physical parameters can determine the sign, + or −, of the response.

There is also clear suggestion from the present results that the concurrent character of ambient, subtle geophysical parameters can determine the character of the short-range, field interactions between adjacent organisms, in separate containers. These effective ambient-field differences can be as small as those associated with relative motions of earth and moon.

The presence of a quarterly lunar variation either alone, or concurrently with a larger unimodal or biomodal one has been evident in previously published results including responses to light in guppies (Lang, 1965, 1972) and planarians (Brown and Park, 1967a) and oxygen consumption and spontaneous motor activity of a variety of kinds of organisms (Brown, 1965). Indeed, in an unpublished full-year study in our laboratory a mean lunar quarterly variation in phototactic response in the flatworm *Dugesia*, superimposed on a larger-ranged unimodal component, has been confirmed during 1972–73. Minima in response occurred one to two days prior to the lunar quarters; the mean range from minima to maxima for the four mean cycles averaged 12.8% increase, strikingly of the same degree as for bean water uptake.

An apparent propensity for inversions of parts or whole of lunar cycles has been described (Brown, 1960, 1965). It seems reasonable to postulate, therefore, that existence of the quarterly lunar response component is widespread among both plants and animals. The potential for one or more of the quarterly components to undergo inversions in response to still undisclosed conditions has probably been responsible for the great difficulty experienced by previous investigators of lunar variations in organisms to repeat at will either their own results or those obtained by others. The current results, however, demonstrate a continuing, substantial periodic lunar influence upon a living system.

Described correlations of organismic variations with primary cosmic radiation (Brown, Webb and Bennett, 1958), with day-to-day geomagnetic fluctuations (Stutz, 1971), and with known geomagnetic mean cyclic patterns (Lang, 1972; Brown and Park, 1967b) suggest that the effective parameters providing the periodicities will be found within the electromagnetic family of forces. The evidence to date points to a probable simultaneous influence of several of their many parameters, and suggests that the ultimate solution will be neither quick nor simple.

These observations demonstrate that in what has been hitherto widely presumed to be fully equivalent environmental conditions there can be substantial rate differences. These differences appear to be accountable in terms of (1) a continuing organismic response to some subtle pervasive, and normally uncontrolled, geophysical variations and (2) the capacity of living systems to alter their sign of correlating state. Discovery of ways to regulate or modify these two response behaviors has potential practical applications, especially if the quarterly lunar influences and occurrence of + and − states prove to be as widespread as present indications now suggest and to include man himself.

In conclusion, the phenomenon of water uptake by such seeds as beans appears to provide one of the simplest of presently known means of investigating—in terms of organism, methods, and equipment—interactions of living systems with their subtle and pervasive geophysical environment, their characteristics, mechanisms, and roles.

The authors gratefully acknowledge the assistance of Mr. Jack S. Pierce in obtaining the data of series G, H and I.

Summary

1. Rate of water uptake by bean seeds during the initial four hours displays a significant quarterly lunar variation.

2. Under what appear to be minimally distrubed environmental conditions relative to environmental electromagnetic fields, maximum rates tend to occur close to new and full moon and the moon's quarters.

3. One or more of the quarterly cycles may undergo periods of inversion either apparently "spontaneously" or in response to such experimentally altered environmental conditions as those found within a walk-in constant-temperature chamber, or effected by very slow uniform rotation.

4. The character of an interaction between vessels of beans located close to one another displays a synodic monthly variation. A maximum in interaction-induced *negative* correlation between two samples occurs 4 to 5 days after full moon, and in *positive*, 4 to 5 days after new moon.

5. These results give further support for the hypothesis that living systems can exist in either of two states, + and − with respect to their correlation with fluctuating biologically effective and normally uncontrolled, pervasive geophysical parameters, and that this sign is experimentally alterable.

LITERATURE CITED

Brown, F. A., Jr., 1960. Response to pervasive geophysical factors and the biological clock problem. *Symp. Quant. Biol., Cold Spring Harbor,* 2: 57–71.

Brown, F. A., Jr., 1965. A unified theory for biological rhythms: Rhythmic duplicity and the genesis of 'circa' periodisms. Pages 231–261 in Ed. J. Aschoff, *Circadian Clocks.* North Holland Pub. Co., Amsterdam.

Brown, F. A., Jr., and C. S. Chow, 1973. Interorganismic and environmental influences through extremely weak electromagnetic fields. *Biol. Bull.,* **144**: 437–461.

Brown, F. A., Jr., and Y. H. Park, 1967a. Association-formation between photic and subtle geophysical stimulus patterns—a new biological concept. *Biol. Bull.,* **132**: 311–319.

Brown, F. A., Jr., and Y. H. Park, 1967b. Synodic monthly modulation of the diurnal rhythm of hamsters. *Proc. Soc. Exp. Biol. Med.,* **125**: 712–715.

Brown, F. A., Jr., H. M. Webb and M. F. Bennett, 1958. Comparison of some fluctuations in cosmic radiation and in organismic activity during 1954, 1955, and 1956. *Amer. J. Physiol.,* **195**: 237–243.

Bryant, T. R., 1972. Gas exchange in dry seeds: circadian rhythmicity in absence of DNA replication, transcription and translation. *Science,* **178**: 634–636.

Lang, H. J., 1965. Übereinstimmungen im Verlauf lunarer Rhythmen bei verschiedenartigen biologischen Vorgängen. *Naturwissenschaften,* **13**: 401.

Lang, H. J., 1972. Korrelation und Kausalität bei lunaren Periodizitätserscheinungen in Biologie und Geophysik. *Nachr. Akad. Wiss. Göttingen, Math.-Phys. Kl.,* **8**: 30–34.

Stutz, A. M., 1971. Effects of weak magnetic fields on Gerbil spontaneous activity. *Ann. New York Acad. Sci.,* **188**: 312–323.

Reference: *Biol. Bull.*, **145**: 279–291. (October, 1973)

A NEW THEORY ON THE MECHANICS OF CILIARY AND FLAGELLAR MOTILITY. I. SUPPORTING OBSERVATIONS[1]

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Over the course of several years, observations on negatively stained and living cilia and sperm flagella from a number of different sources have been accumulated in this laboratory. Collectively, these findings have provided growing evidence in favor of a new and relatively simple explanation of the mechanics of motility in such organelles, which was outlined in a very preliminary form earlier (Costello and Henley, 1970). In the present paper, the supporting data will be examined; the new theory, and the relation of these data to it are outlined in a second communication (Costello, 1973).

In general, the evidence indicates that under certain conditions, doublet microtubules bend into coils or helical configurations, while at the same time the singlets straighten, or stiffen and remain straight for a finite period. I believe that these phenomena are directly related to the mechanism of ciliary and flagellar motility.

MATERIALS AND METHODS

The biological material consisted of a number of invertebrates, principally freshwater and marine flatworms. The freshwater material was collected from streams in the vicinity of Chapel Hill, North Carolina, and from Stone Mountain, Georgia (*Mesostoma georgianum*). The marine material was furnished by the Supply Department of the Marine Biological Laboratory at Woods Hole, Massachusetts, or collected at Pacific Grove, California (*Polychoerus carmelensis*). A great deal of this material was prepared for another purpose and studied largely by Dr. Catherine Henley, to whom I am greatly indebted, both for the opportunity of examining her extensive collection of electron micrographs and for the use of a number of these for measurements and to illustrate this paper.

The material examined included the following: the marine acoels *Polychoerus carmelensis, P. caudatus, Childia spinosa* (*groenlandica?*) and *Anaperus gardineri;* the freshwater rhabdocoels *Mesostoma georgianum, Microdalyellia* sp. and *Macrostomum* sp.; the marine alloeocoels *Monoophorum* sp., *Monocelis* sp. and *Plagiostomum* sp.; the triclads *Dugesia tigrinum* (freshwater) and *Bdelloura candida* (marine); the marine polyclads *Stylochus zebra* and *Notoplana* sp.; the freshwater rhynchocoel *Prostoma rubrum;* the marine polychaete annelid *Chaetopterus pergamentaceus;* the earthworm *Lumbricus terrestris;* the prosobranch freshwater mollusc *Goniobasis proxima;* and several unidentified polyclads collected off the North Carolina coast.

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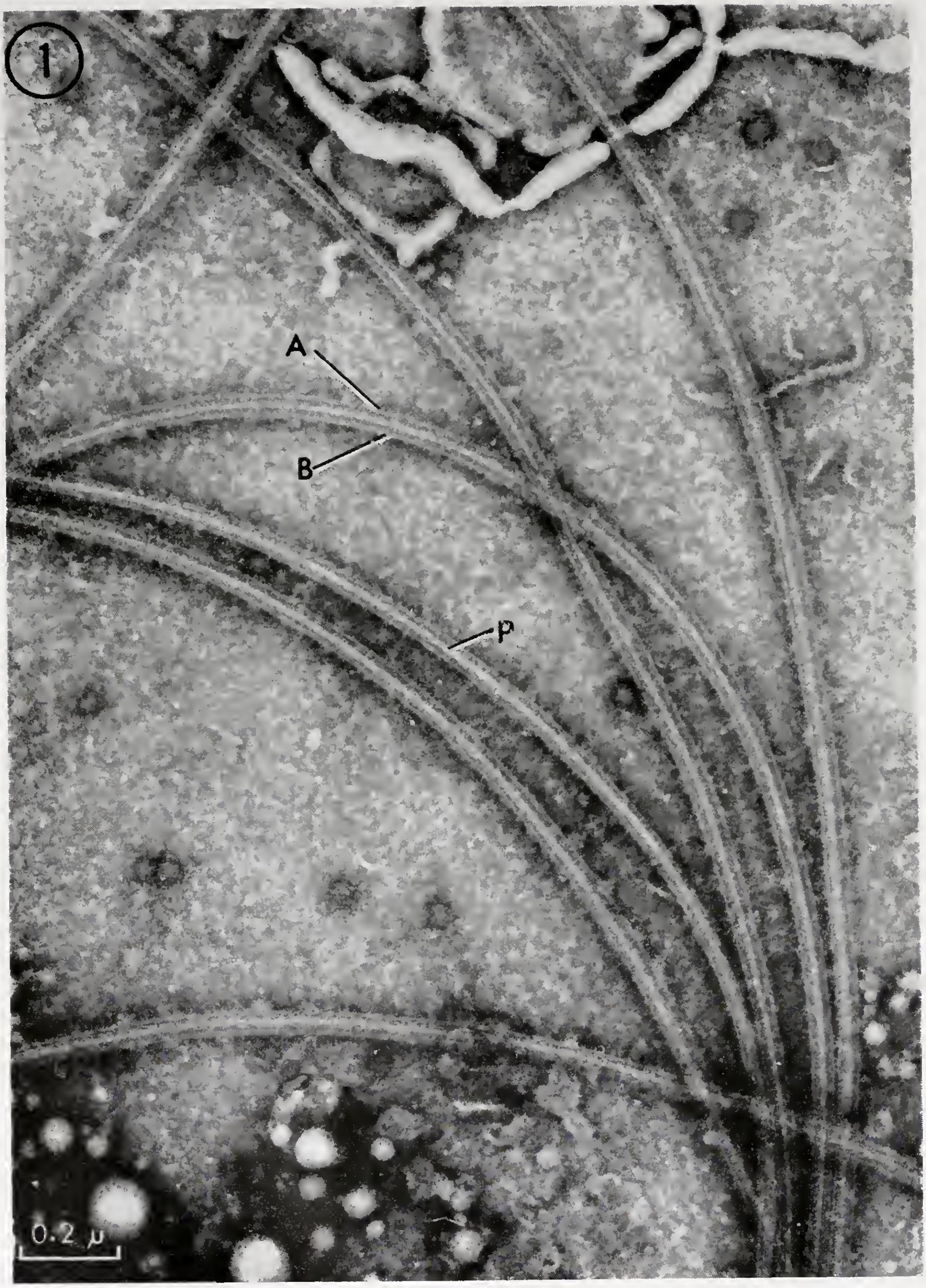


FIGURE 1. Part of a group of doublet microtubules of an isolated cilium of *Mesostoma*, showing relation of bending to the A- and B-subtubules (A, B) and partition wall (P) between them. See text; 70,000 ×.

The phosphotungstate negative staining (1% PTA, pH 6.8) of the spermatozoa, of earlier stages of spermiogenesis, and of the surface cilia was supplemented by study of material fixed, sectioned and stained for electron microscopy, and by very extensive studies by ordinary light and phase contrast microscopy of living and fixed cilia and spermatozoa, both normal and PTA-treated. For details of techniques, see Costello, Henley and Ault (1969), Henley, Costello, Thomas and Newton (1969), Henley (1970a) *etc.* An extensive pretreatment of earthworm spermatozoa with distilled water, prior to PTA maceration and negative staining, was used in demonstrating that the singlets are attached to each other (Henley, 1970b) and show exceptionally rigid elastic properties; see Henley (1973) for details.

All micrographs were made with the Zeiss 9A or 9S2 electron microscope.

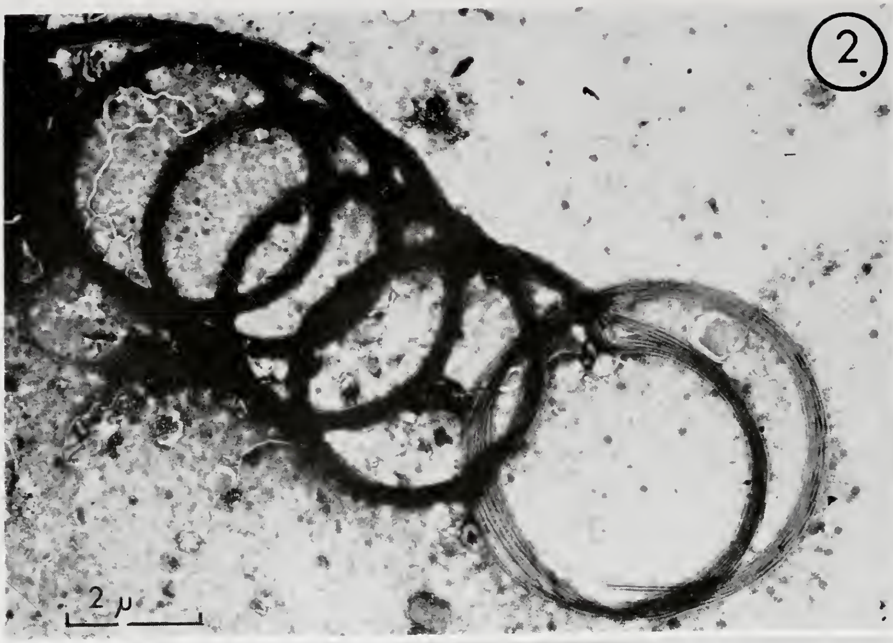
OBSERVATIONS

Evidence for coiling and straightening

Evidence that induced coiling of the doublets is of importance in the interpretation of the bending process in ciliary and flagellar motility is supplied by observation of isolated groups of axonemes (Figs. 1–6; see also Henley *et al.*, 1969), and analysis of selected electron micrographs, such as Figures 7 and 8.

From the arrangement of the coils of the doublets, in relation to the partition wall between subtubules A and B, and from the location, on the coils, of the partially macerated spokes, it is concluded that the bending of the doublet occurs in a direction at right angles to the partition wall, and away from the region where the dynein arms were attached (see Fig. 1 in the following paper). That is, the flexible, tree-like remnants of the spokes, which are attached at regular intervals to the A-subtubule, extend out on the convex side of the flattened doublet coils (Fig. 6 and, more strikingly, in Henley *et al.*, 1969, Fig. 4, page 853). The partition wall is most often perpendicular to the substratum and one side of both the A- and the B-subtubule is in contact with the grid coating (Fig. 1). In addition, the A-subtubule, which forms a complete circle, shows up as wider than the B-subtubule, which is incomplete. So far as I can ascertain from the literature, investigators considering modes of axonemal motility have not realized that the doublets must do their active bending in a particular plane in relation to their doublet structure. It is a matter of simple logic to assume that the mode of active bending of doublets is dependent upon their ultrastructural organization, including the location of the ATPase, and that because of this organization, they cannot bend equally easily in all planes. If one is thinking in terms of a sliding filament theory as the basis of movement (Satir, 1965, 1968; Summers and Gibbons, 1971; Sleigh, 1973), in axonemal motility there must be a bending of the filaments accompanying the sliding of filaments upon one another (see also Brokaw, 1971, 1972). Mere sliding of straight, unhampered microtubules upon each other could produce only a jack-in-the-box effect at their tips. Horridge (1965) provided evidence for both sliding and bending of doublets in the macrocilia of *Beroë*, but it was uncertain at that time how this might apply to single axonemes.

Negatively stained preparations made by macerating cilia to the point where the membrane and matrix have been removed but with the doublets still attached to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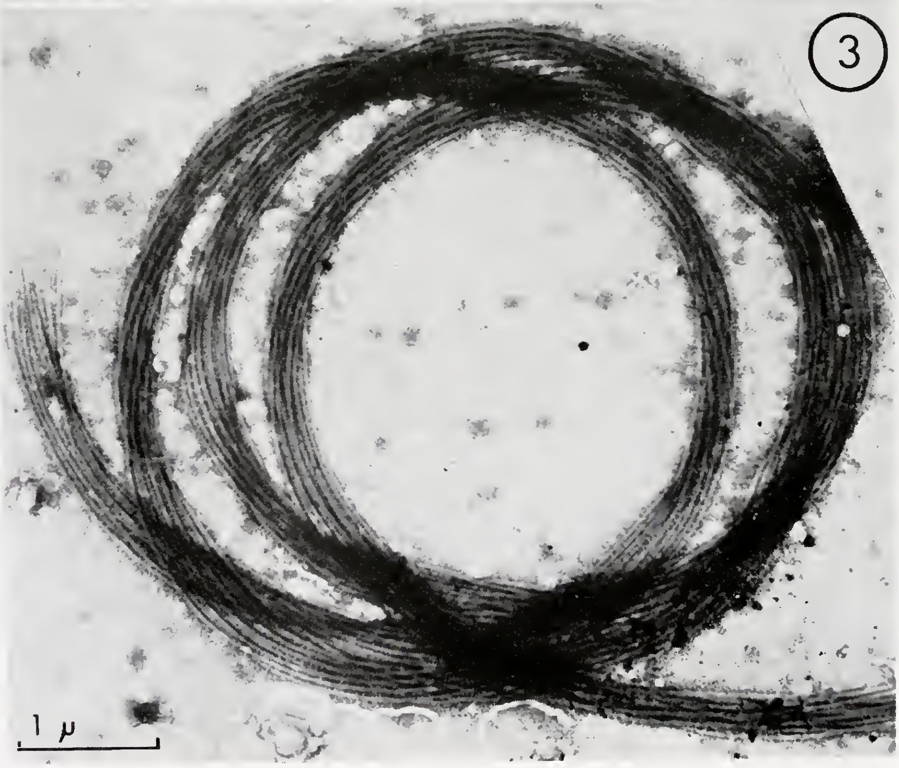
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the basal plate show the doublets flared out in 9 arcs which do not close to form complete circles (Figs. 7, 8). Note that in each of these cases, four of the doublets bend to one side, and five in the opposite direction. The central singlets in Figure 7 are in the stiffened condition and in Figure 8 they are in the relaxed condition. After an appropriate amount of maceration, such configurations are common. The overall picture is much like the diagram of the form or positions of a cilium during various effective and recovery portions of the stroke. The lengths measured along these ciliary doublets for a large number of turbellarians were 12 to 15 microns. On the other hand, axonemal isolates of long flagella macerated to a somewhat lesser degree show repeated coiling into circles of quite uniform diameter, usually averaging from 3 to 4.5 microns (see Figs. 2 and 3). The circumference of a single coil of a flagellar doublet thus corresponds approximately to the length of a doublet of a ciliary axoneme. On the basis of these comparisons, a complete arc or a single coil would correspond to the length of axoneme involved in $\frac{1}{2}$ wavelength, two coils to that of a whole wavelength.

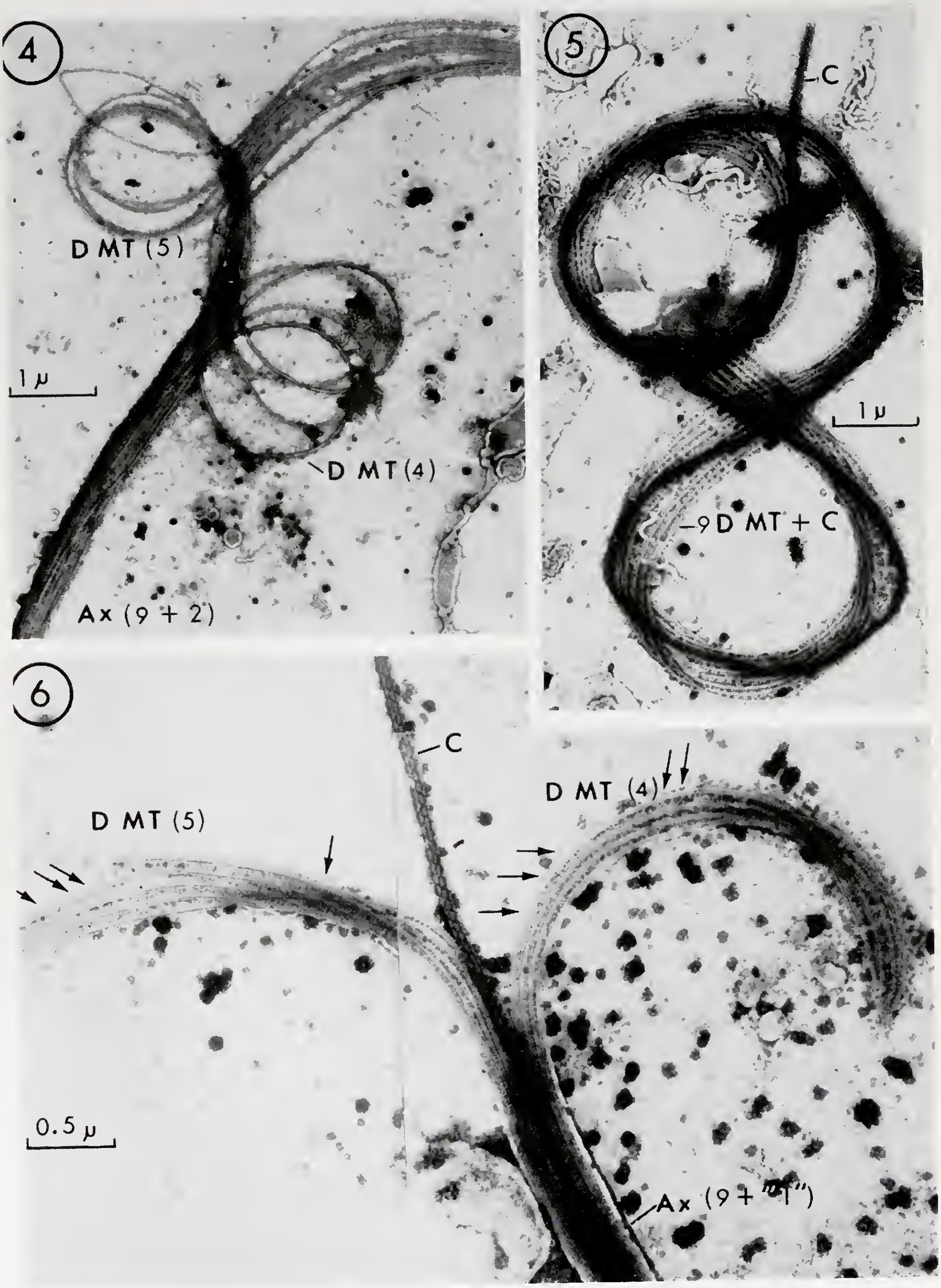
In "9 + 1" flagella, if maceration with the phosphotungstate is of insufficient duration to free the central core, this structure remains encompassed by the doublets and bends as the axoneme coils. Under these circumstances (which involve, also, incomplete matrix removal) the coils may be of varying diameters. With a greater degree of maceration, all or part of the central core "escapes" from the doublets (Figs. 5 and 6) and is very frequently found extending along a relatively straight course for considerable distances—as much as 73 microns in one case (Henley *et al.*, 1969).

A number of interesting configurations have been observed after PTA treatment of various spermatozoa. For example, Figure 4 depicts twin looping in a partially macerated axoneme of a spermatozoon of *Chaetopterus*. The portion of the axoneme (Ax) nearer the basal plate is less macerated than the more distal portion, and the looping has occurred at the junction of the two regions. That the "clockwise" (4 doublets) and "counterclockwise" (5 doublets) loops are related to the two lateral half doublet groups (with doublets #5 and #6 presumably in the latter) is a reasonable certainty.

Occasionally there are 6 doublets bending to one side and only three to the other in both the isolated macerated cilia and flagellar tips. In these cases the six were often seen to consist of one group of four and another group of two doublets, with the latter identifiable as the #5–6 pair. The more usual pattern of four doublets bending to one side while five doublets bend to the other side is thus logically modified.

Figure 5 is an electron micrograph of a portion of a "9 + 1" axoneme of a PTA-treated spermatozoon of *Dugesia*. The core is included within the coils until it becomes free. At this point the doublets continue their bent course and the fragment of escaped core is straight. The significance of the figure-of-eight, with "clockwise" and "counterclockwise" bends of the axoneme, is probably related to the two lateral half-groups of doublets bending in opposite directions. Figure 6 is a similarly treated portion of a spermatozoan axoneme of *Dugesia*, at the point

FIGURES 2 and 3. PTA-treated sperm axonemes of *Goniobasis*, showing induced coiling. See text; 8900 × and 18,600 ×, respectively.


4
D MT (5)
1 μ
D MT (4)
Ax (9 + 2)
5
C
1 μ
9 D MT + C
6
C
D MT (4)
D MT (5)
0.5 μ
Ax (9 + "1")

FIGURES 4, 5 and 6. Partially macerated PTA-treated 9 + 2 sperm axoneme of *Chaetopterus* (Fig. 4) and "9 + 1" sperm axonemes of *Dugesia* (Figs. 5 and 6). See text; Figure 4, of escape of the straight core from the doublets. There are 4 doublets bending "clockwise" and 5 doublets bending "counterclockwise." All have remnants of spokes visible on their *convex* surfaces.

Identification of doublets by number

In certain electron micrographs of ciliary isolates that had been optimally macerated and negatively stained, the #5–6 attached doublet pair could be identified with considerable certainty. In addition, the points of insertion of all the doublets into the basal plate could sometimes be seen. Enlargement of these micrographs and careful examination made possible identification of the doublet microtubules by number. Figure 8 is the best example of this, and all doublets could be identified in Figure 7, also.

Figure 8 shows a cilium of *Mesostoma georgianum*, with the #5–6 doublets demonstrably attached to one another over part of the basal two-thirds of their lengths, and flared apart in opposite directions distally. This suggests that, in this form at least, the dynein arms-back bridge connectives are quite stable, and not totally disrupted or macerated by the PTA treatment. Since the #5–6 doublets were readily identifiable, it was possible to identify also, by number, every other individual doublet, and to correlate their identities with the direction of their bending. Thus far, from this and other examples, the data are consistent with the view that in cilia the doublets of one lateral half (#2, #3, and #4) bend in a direction opposite to that of the doublets of the other lateral half (#7, #8 and #9). With little maceration and with the matrix intact the axoneme bends as one unit.

Induced coiling of glutaraldehyde-fixed spermatozoa

Additional evidence that PTA induces sperm flagella to become helically coiled has been obtained. Spermatozoa of the marine annelid *Chaetopterus* were taken from the parapodia of a male and fixed in a cacodylate-buffered glutaraldehyde fixative devised for acoel flatworms. The spermatozoa did not coagulate into a mass, but were fixed, in suspension, essentially in a straight form. Diluting the glutaraldehyde-fixed spermatozoa with buffer-sucrose wash did not alter this form (Fig. 9). Diluting the glutaraldehyde-fixed spermatozoa 1:4 with 1% PTA at pH 6.8, however, induced the straight tails to assume strikingly helical patterns. Observation by phase contrast microscopy revealed that the tails of the intact fixed spermatozoa became curved, looped, and then helical, with up to three gyres, in the course of 20 to 30 minutes (Fig. 10). Four per cent PTA at pH 2, mercuric chloride and lead nitrate solutions also induced excellent spiralling of the tails. It is of interest that the form of the "wave" was clearly not planar. The changes occurred so slowly in these dead, fixed spermatozoa that actual movement of the flagellum was not perceptible. While I have not been able to reverse the coiling with specific agents, the helices are not rigid. The tails straighten out readily when a directed flow of the surrounding medium is induced under the coverglass.

11,665 ×; Figure 5, 11,665 ×; Figure 6, 23,750 ×; Ax, axoneme; C, core; D MT, doublet microtubules.

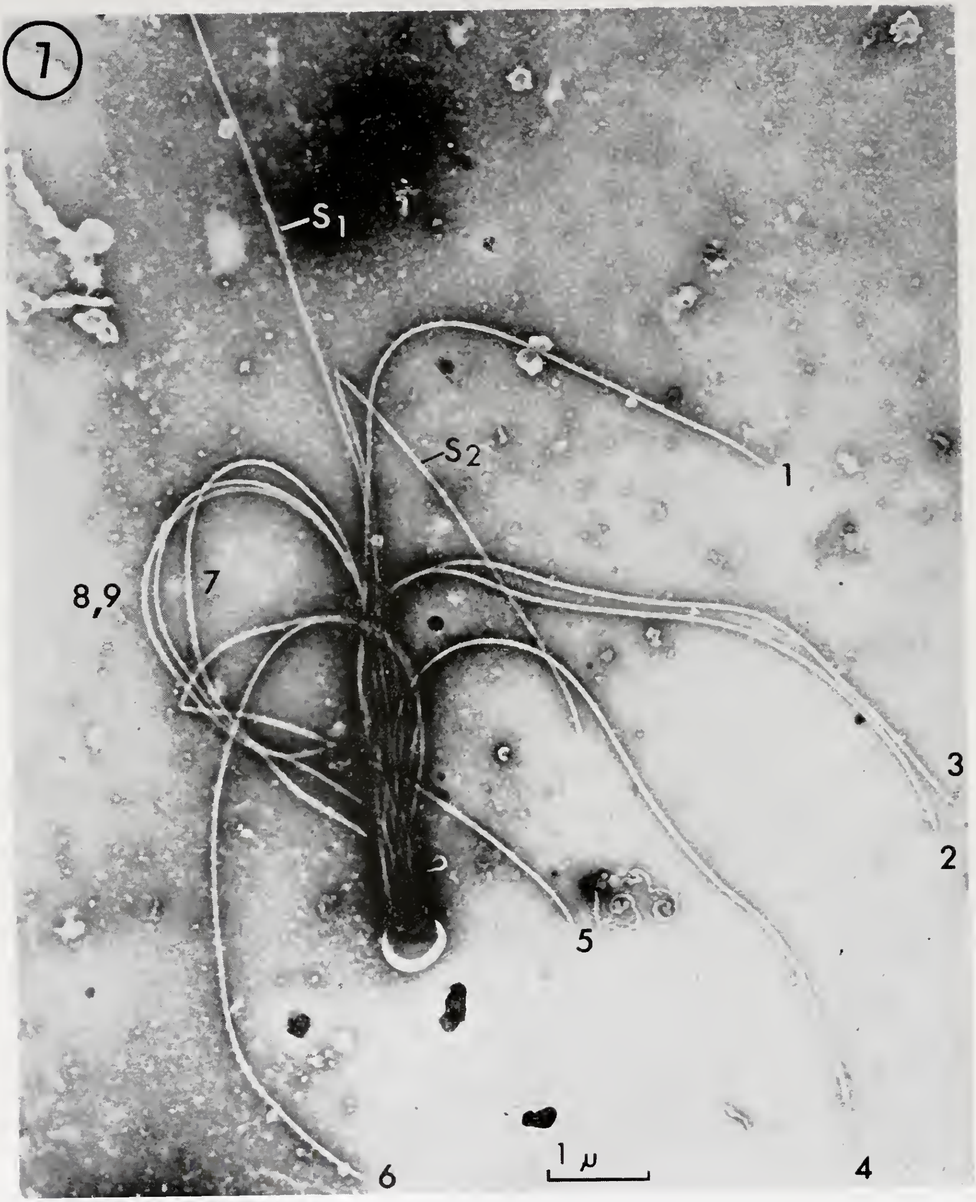


FIGURE 7. Macerated, negatively stained (by PTA) surface cilium of *Mesostoma*, with bent doublets (identified by number) and straight singlets (S_1, S_2). Doublets #1–4 bend to right, #5–9 to left; doublets #5 and #6 attached over part of length; 13,250 ×.

These results have been reported, thus far, only in abstract (Costello and Henley, 1969).

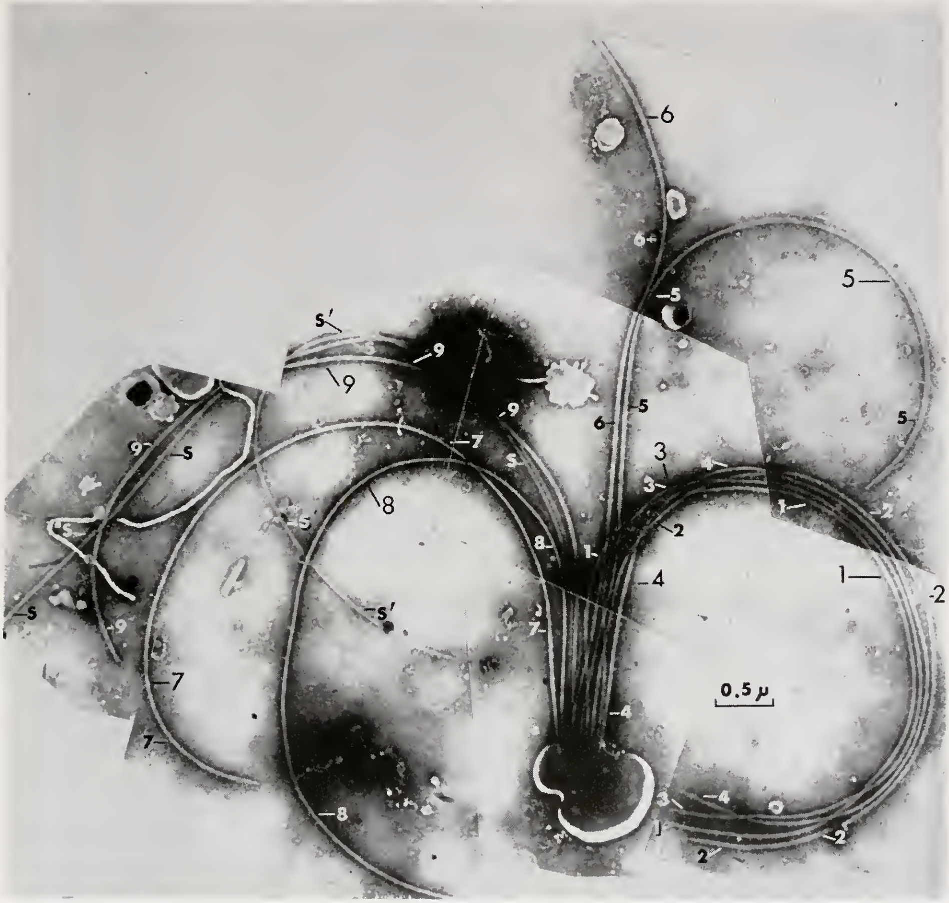
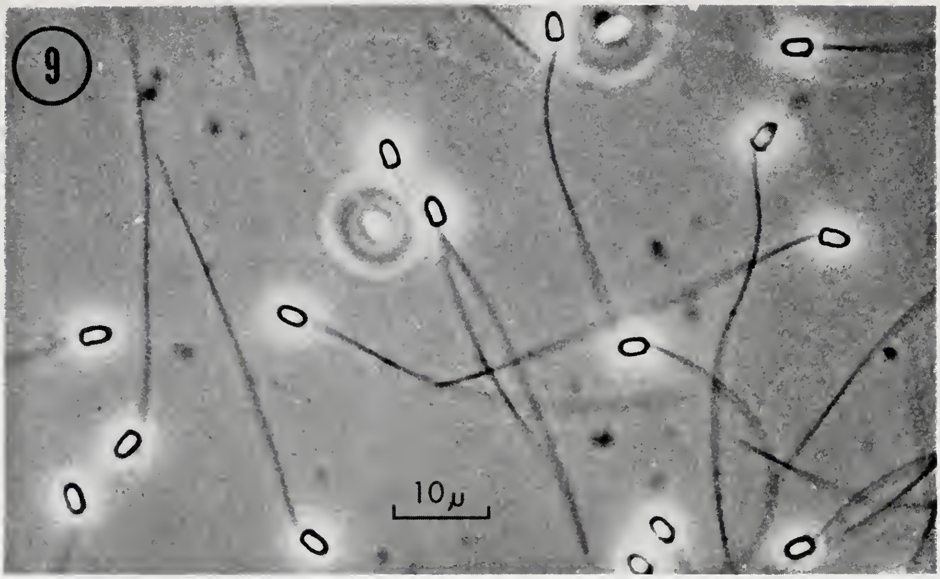


FIGURE 8. Macerated negatively stained (by PTA) surface cilium of *Mesostoma* with clearly identifiable doublets and singlets S and S′. Doublets #1–5 bend to right, and #6–9 to left. Each doublet is identified by a large number, plus smaller numbers to trace its course; 15,390 ×.

Rigidity of central singlet microtubules in earthworm sperm

The spermatozoon of the earthworm has what superficially appears to be the conventional 9 + 2 pattern of microtubules. However, the two central singlets can be seen, in both sectioned and negatively stained material, to be connected to one another by short bridges at regular intervals, and to be accompanied by longitudinal paired fibrous elements which are clearly solid and smaller in diameter than microtubules (Henley, 1973). Under certain conditions of negative staining this complex of cross-linked singlets plus paired fibrous elements is found to fall in configurations similar to those seen in negatively stained "9 + 1" cores. The evidence suggests that the central complex in earthworm spermatozoa has elastic properties very different from those exhibited by the doublets, and that this com-



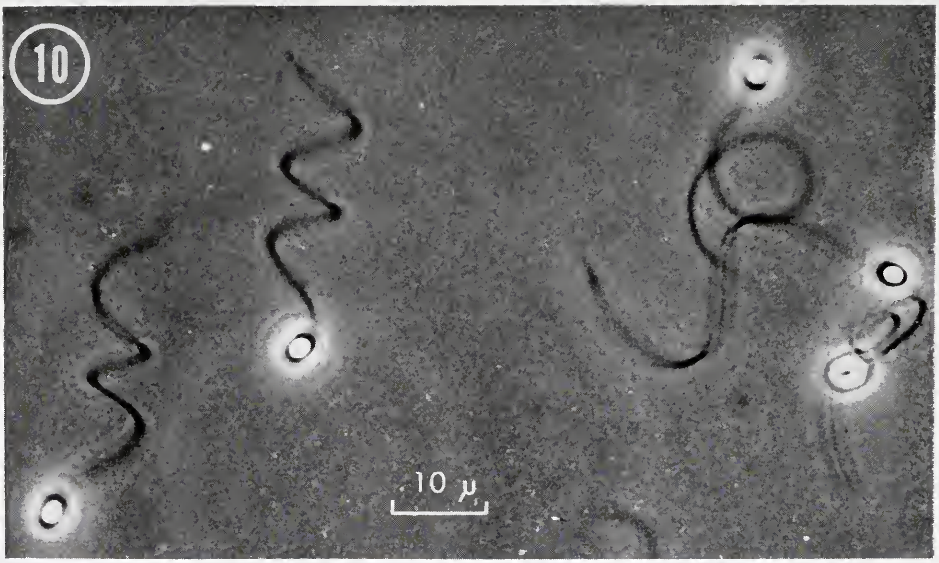


FIGURE 9. *Chaetopterus* spermatozoa fixed in cacodylate-buffered glutaraldehyde. Suspension was later diluted with buffer wash. Note essentially straight tails; phase-contrast, 1280 ×.

FIGURE 10. *Chaetopterus* spermatozoa fixed in cacodylate-buffered glutaraldehyde, and later diluted with 1% PTA, pH 6.8. Note helically coiled tails; phase-contrast, 1280 ×.

plex is clearly involved in a stiffening function. The connectives could inhibit or prevent the sliding of singlets, relative to each other, that would be expected to accompany lateral bending. Gibbons (1961) found sometimes single, sometimes double bridges between the central singlets of lamellibranch cilia.

During the course of the present investigation we have found clear-cut evidence for regularly repeated connectives between the central singlets of the cilia of *Hydrolimax grisea,* also.

Helical-protofibrillar transitions

Another possibly pertinent series of observations relates to the transitions between the protofibrillar arrangement and a helical configuration of subunits in the microtubules. Such transitions were observed by Henley in negatively stained *Mesostoma* sperm in 1969, and described in detail by Thomas (1970) for PTA-treated *Stylochus* sperm and by Thomas and Henley (1971) for spermatozoa of *Macrostomum.* Transitions were found both in cortical singlet and in doublet microtubules. The singlets, which characteristically have a helical arrangement of the subunits, often showed a transition to the protofibrillar condition. Thomas (1970, page 231), states that neither the protofibrillar nor the helical configuration appears to be characteristic of the intact unmacerated doublets, but that both conditions were to be found in her PTA-treated material. However, extended observations on a number of different species of spermatozoa make it increasingly clear that the protofibrillar condition is characteristic of the straight, intact, PTA-treated doublet. But, transitions do occur.

If subtubule B (the incomplete subtubule) is macerated away, leaving subtubule A (the complete member), then the subunits of A assume a helical arrangement (Thomas, 1970, Figs. 12–15). Similar transitions are found in subtubule A of earthworm axonemal doublets when the B member is macerated away (see Fig. 9 in Henley, 1973).

The evidence suggests that singlets and doublets, in their "normal" states, exist in opposite phase—that is, singlets with subunits arranged in the helical state and doublets with subunits in the protofibrillar state.

Cohen, Harrison and Stephens (1971) have obtained x-ray diffraction patterns of wet gels of A-subtubules from sea urchin sperm-tail doublets. The results indicate that subunits with a 40–50 Å packing diameter form filaments, alternately half-staggered, parallel to the tubule's axis. A 12- or 13-stranded structure best fits the x-ray diagram. A dried A-subtubule sample gives an x-ray diffraction pattern with a strong meridional reflection at 40 Å, rather than the off-meridional diffraction seen with native specimens. The lateral bonds of the surface lattice of the A-subtubule are destroyed by drying, leaving the stronger axial bonds connecting simple linear arrays of subunits (see, also, Stephens, 1973).

Bearing in mind the fact that A-subtubules may differ from intact doublets (as discussed above), the evidence is compatible with the idea that a conformational change in subunit arrangement may be involved in microtubule bending. The possible implications of all these observations will be considered in the next paper, in relation to a theory which attempts to account for the mechanics of ciliary and flagellar motility.

SUMMARY

1. Controlled partial maceration and negative staining of ciliary and flagellar axonemes (with phosphotungstate) have revealed that under these conditions: (a) Doublet microtubules have an inherent tendency for bending or coiling; (b) Central singlet microtubules of 9 + 2 axonemes, or the cores of the "9 + 1" flagellar axonemes, stiffen or straighten; (c) Active bending of the doublets occurs in the direction away from their dynein arms, so that the A-subtubule is on the convex side.

2. In partially macerated 9 + 2 axonemal isolates, examination of electron micrographs indicates that the bending of the doublets of one lateral half is in a direction opposite to that of the doublets of the other lateral half. This is a consequence of (1, c) above.

3. Evidence is presented for (2) above, in micrographs showing the direction of bending of specifically identified doublet microtubules of isolated ciliary axonemes.

LITERATURE CITED

BROKAW, C. J., 1971. Bend propagation by a sliding filament model for flagella. *J. Exp. Biol.*, **55**: 289–304.

BROKAW, C. J., 1972. Flagellar movement: a sliding filament model. *Science*, **178**: 455–462.

COHEN, C., S. C. HARRISON AND R. E. STEPHENS, 1971. X-ray diffraction from microtubules. *J. Mol. Biol.*, **59**: 375–380.

COSTELLO, D. P., 1973. A new theory on the mechanics of ciliary and flagellar motility. II. Theoretical considerations. *Biol. Bull.*, **145**: 292–309.

COSTELLO, D. P., AND C. HENLEY, 1969. Straightening, bending, and spiraling in glutaraldehyde-fixed spermatozoa: a reactivation phenomenon. *Biol. Bull.*, **137**: 397.

COSTELLO, D. P., AND C. HENLEY, 1970. A simple basic theory to account for the difference between ciliary and flagellar motility. *J. Cell Biol.*, **47**: 42a.

COSTELLO, D. P., C. HENLEY AND C. R. AULT, 1969. Microtubules in spermatozoa of *Childia* (Turbellaria, Acoela) revealed by negative staining. *Science*, **163**: 678–679.

GIBBONS, I. R., 1961. The relationship between the fine structure and direction of beat in gill cilia of a lamellibranch mollusc. *J. Biophys. Biochem. Cytol.*, **11**: 179–205.

HENLEY, C., 1970a. Changes in microtubules of cilia and flagella following negative staining with phosphotungstic acid. *Biol. Bull.*, **139**: 265–276.

HENLEY, C., 1970b. Ultrastructure of the negatively stained spermatozoon of the earthworm. *Biol. Bull.*, **139**: 403.

HENLEY, C., 1973. Ultrastructure of the spermatozoon of the earthworm as revealed by negative staining. *J. Morphol.*, **140**: 197–214.

HENLEY, C., D. P. COSTELLO, M. B. THOMAS AND W. D. NEWTON, 1969. The "9 + 1" pattern of microtubules in spermatozoa of *Mesostoma* (Platyhelminthes, Turbellaria). *Proc. Nat. Acad. Sci., U. S.*, **64**: 849–856.

HORRIDGE, G. A., 1965. Macrocilia with numerous shafts from the lips of the ctenophore *Beroë*. *Proc. Roy. Soc. London, Series B*, **162**: 351–364.

SATIR, P., 1965. Studies on cilia. II. Examination of the distal region of the ciliary shaft and the role of the filaments in motility. *J. Cell Biol.*, **26**: 805–834.

SATIR, P., 1968. Studies on cilia. III. Further studies on the cilium tip and a "sliding filament" model of ciliary motility. *J. Cell Biol.*, **39**: 77–94.

SLEIGH, M. A., 1973. Cell motility. Pages 525–569 in E. E. Bittar, Ed., *Cell Biology in Medicine*. John Wiley & Sons, New York, London.

STEPHENS, R. E., 1973. Enzymatic and structural proteins of the axoneme. In press in M. Sleigh, Ed., *Cilia and Flagella*. Academic Press, New York.

SUMMERS, K. E., AND I. R. GIBBONS, 1971. Adenosine triphosphate-induced sliding of tubules in trypsin-treated flagella of sea-urchin sperm. *Proc. Nat. Acad. Sci. U. S.*, **68**: 3092–3096.

THOMAS, M. B., 1970. Transitions between helical and protofibrillar configurations in doublet and singlet microtubules in spermatozoa of *Stylochus zebra* (Turbellaria, Polycladida). *Biol. Bull.,* **138**: 219–234.

THOMAS, M. B., AND C. HENLEY, 1971. Substructure of the cortical singlet microtubules in spermatozoa of *Macrostomum* (Platyhelminthes, Turbellaria) as revealed by negative staining. *Biol. Bull.,* **141**: 592–601.

Reference: *Biol. Bull.,* **145**: 292–309. (October, 1973)

A NEW THEORY ON THE MECHANICS OF CILIARY AND FLAGELLAR MOTILITY. II. THEORETICAL CONSIDERATIONS[1]

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Evidence presented in the preceding paper (Costello, 1973) strongly suggests certain characteristics of axonemal microtubules. (1) Doublet microtubules have an inherent tendency towards bending or coiling, the expression of this tendency being governed by the length of the axoneme. (2) Central singlet microtubules of the 9 + 2 configuration, or the core of the "9 + 1" pattern, in contrast, become stiff or straight under certain conditions. (3) Active bending of the doublets is in the direction away from their dynein arms, so that the B-subtubule is on the concave side. These observations may all be correlated into a unifying hypothesis to account for the mechanics of ciliary and flagellar motility.

An earlier theory, along somewhat the same lines, was that of Bradfield, (1955), who treated all axonemal components as single units. At that time, all that was known of the substructure of the peripheral "fibers" was that there were two "subfibers" per peripheral "fiber" in cilia and many flagella. However, even this fact was ignored by Bradfield in the formulation of his theory. A reconsideration of these older ideas is therefore highly desirable.

THEORY

General aspects

The theory depends upon certain assumptions and corollaries, which follow:

(1) Both singlet and doublet axonemal microtubules can exist in either the activated or the relaxed state. In the activated state, the doublets bend, while the singlets straighten and stiffen, and the bending or straightening waves are propagated along the respective types of microtubules, at different rates and with differing durations.

(2) We assume that the resistance to bending of an axoneme due to stiffening of the central singlets is greatest in the plane connecting the axes of the singlets where they are fastened together and thus reinforce each other, and least in the direction at right angles to this plane. Partly because there are more doublets than singlets, we assume also that the active bending of three doublets in this latter direction is quite sufficient to overcome the resistance and produce basal bending of the stiffened singlets and of the intact cilium.

(3) In the relaxed condition, both types of microtubules may possess some degree of structural rigidity rather than being completely limp. In addition, they are held in normal association to each other by the matrix of the organelle, by the

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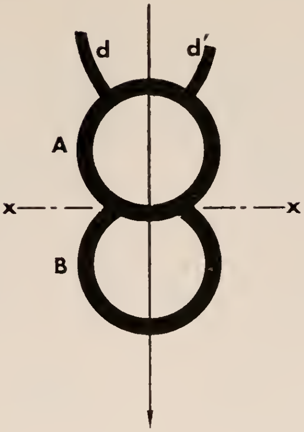


FIGURE 1. Diagram of doublet microtubule, showing direction of bending (arrow) at right angles to axis X--X; A = complete microtubule; B = incomplete tubule; d, d′ = dynein arms.

"spokes" (radial connectives between doublets and central structures), by the connectives to the plasma membrane from the doublets, by the nexin links (circumferential connectives) between the A-subtubules (Stephens, 1970), and by the bridges between the singlets of the central pair, where these exist. In this relaxed state, non-activated microtubules of the axoneme can be moved passively in any direction by those doublet microtubules which become activated and bend.

(4) It is assumed that the waves propagated out the doublets are initiated successively, in unidirectional order, by impulses arising at or under the basal plate. The activated doublets bend in a direction dictated by their ultrastructural organization, namely, in a direction away from their dynein arms (Fig. 1).

(5) Activation of successive doublets, to bend in the directions contributing to either the effective or recovery strokes of a cilium or to the planar or helical movement of a flagellum, is responsible for these types of movement. However, the organelle as a whole moves with its characteristic wave pattern because of its length-wavelength-amplitude relationships and the coordination brought about through integrating these with the behavior of the singlets (or core complex of the "9 + 1" pattern), where these are present, and with the connectives of the axoneme listed above. The bending forces generated in the doublets must act against any normal structural resistance of the relaxed doublets, and against the stiffening forces produced by the central singlets (or the core), as well as against the resistance of the medium. This interaction, in flagella at least, should facilitate unbending, the mechanism of which has always been a problem (Brokaw, 1968; Brokaw, Goldstein and Miller, 1970).

Ciliary motility

This theory will first be applied to an "ideal" simple cilium. An "ideal" cilium is here defined as having an axonemal contour length sufficient for exactly ½ wavelength of beat. A hypothetical commutator-type stimulator, located at or

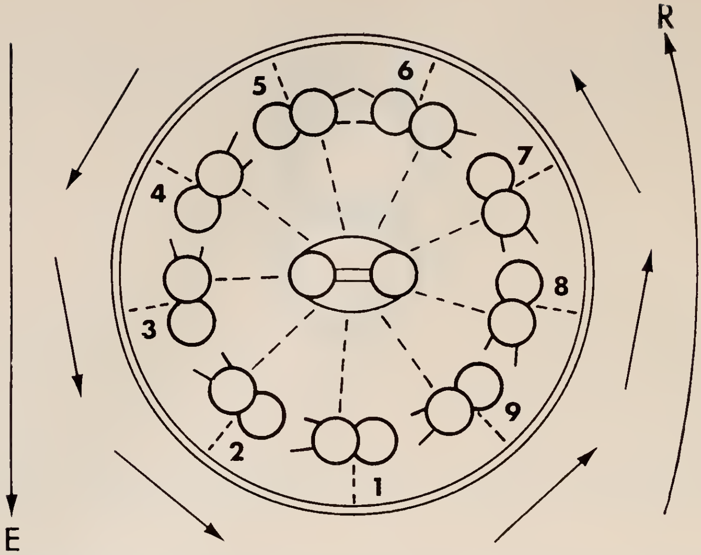


FIGURE 2. Diagram of cross-section of a cilium with doublets numbered #1 through #9; arrow E = direction of effective stroke; arrow R = recovery stroke.

under the basal plate, stimulates the basal end of doublet #1 (as conventionally numbered) to initiate an impulse which will be propagated up this doublet (Figs. 2 and 3E). It is suggested, also, that a stimulus is sent earlier or at the same time to each of the two central singlets, to initiate stiffening impulses which will move up these singlets at a considerably greater rate of propagation. The stimulation of doublet #1 is essentially ineffective in producing any bending in the direction away from its dynein arms because the stiffened singlets are so fastened together by the bridges between them that movement laterally is not possible. This resistance to lateral movement is reinforced by the arrangement of the ciliary rootlets. It is possible, also, that a bending wave moving up a single doublet is insufficient, in itself, to produce much, if any, bending of a cilium as a whole, especially in this direction. A reinforced bending is presumably very much more effective. However, any movement that is produced by doublet #1 would result only in a little basal bending and cause the stiff body of the cilium to incline very slightly to one side (toward doublet #9 of Fig. 2).

The wave stiffening the central singlets would need to travel about four and a half times as fast as the wave propagated to bend a peripheral doublet in order to stiffen the cilium all the way to its tip, by the time the commutator has shifted clockwise to activate doublet #2 to begin its basal bending. This is because of the axonemal length which is a function of $\frac{1}{2}$ the wavelength in the ideal cilium. As the hypothetical commutator travels on to trigger basal bending in doublet #3 and then in doublet #4, the central singlets must be presumed to remain stiff throughout their lengths. While doublets #2, #3 and #4 each bend in the direction away from their dynein arms, for the cilium as a whole the resultant of the bending forces is in the direction toward doublet #1 and away from the

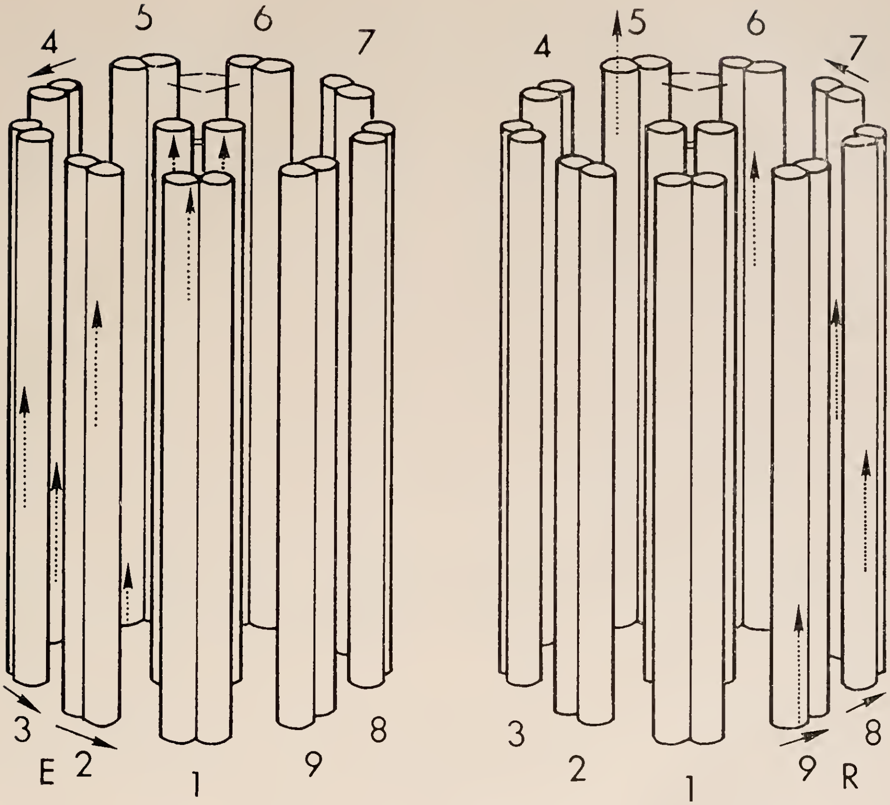


Figure 3. Propagation of waves out microtubules (dotted arrows) and direction of bending (solid, short arrows), for effective stroke (E) and recovery stroke (R), respectively. The dynein arms have been omitted, with the exception of one pair on doublet #5.

bridge connecting doublets #5 and #6 (Arrow E, Fig. 2). Since the central singlets of cilia of most forms do not originate at the basal plate, but at a point some distance (400 Å, at least) above it, basal bending of the cilium as a whole in this plane is not as much inhibited by the stiffened singlets as is the bending of the main shaft of the cilium on out to its tip. The significant factor here is not the distance, which is too little for a bending curve, but the fact that the singlets are not attached to the basal plate. Bending of the singlets in the lateral plane is not feasible because they reinforce each other in maintaining rigidity and cannot slide if they are fastened together. The effective stroke is, therefore, the bending, near the base, of an essentially stiff cilium, brought about by doubts #2, #3 and #4, in the direction of least resistance of the stiffened singlets, *i.e.*, at right angles to the plane connecting their central axes (Figs. 2 and 3E). The doublets which are not stimulated to bend (or singlets when not stimulated to stiffen) are relaxed and can be bent passively.

As the commutator continues to move and activates and then passes doublets

#5 and #6 (Fig. 3R), the central singlets gradually lose their stiffness and become limp progressively, from base to tip. The impulses progressively passing up doublets #2, #3 and #4 have also died out, successively, as they reached the tips of these microtubules. Doublets #5 and #6 are relatively ineffective in producing a lateral movement in the direction of their B-subtubules because (1) they are fastened together and dampen each other, (2) the central pair of singlets is still offering resistance to lateral bending, (3) the ciliary rootlets are so arranged as to inhibit lateral movement, and (4) the effective stroke has already carried the cilium over parallel to the cell surface. So such movement as is produced by attached doublets #5 and #6 will only cause the bent-over cilium to swing slightly out of the plane of the effective beat and somewhat to one side. In fact, doublets #5 and #6 bring the effective beat of the cilium to a complete halt. At the same time as these ineffective impulses terminate at the tips of #5 and #6, the stiffened state of the central singlets is terminated. Thus, when the commutator activates doublet #7 to bend in the direction away from its dynein arms, the recovery stroke is inaugurated. With relaxed central singlets, successive propagation of bending waves up doublets #7, #8 and #9 will produce half of a helical stroke (Figs. 2 and 3R). This is the recovery stroke, with the resultant of forces roughly in the direction opposite that of the effective stroke. This half helical wave will die out as the impulses reach the tips of these fibrils, ending the recovery stroke and completing one wavelength of ciliary beat. If the cilium is beating with little or no interruption, the commutator is again ready to activate the two central singlets and doublet #1 to begin a new beat.

This is, then, a situation where the half wavelength of the effective stroke is not identical with the half wavelength of the recovery stroke, since the two have different forms. What is identical is the length measured along the cilium for both the effective stroke and the recovery stroke, since this is the length of the cilium. (See also Párducz, 1953, 1954, 1961, 1967; Tamm, 1972).

It is assumed that the effective or power stroke carries the cilium from its resting position, whether this is upright or at any other angle, over to where it lies essentially parallel to the ciliated surface. The recovery stroke returns the cilium to its resting position. Therefore, the angle through which the cilium is moved may vary in different forms.

To summarize, ciliary motility consists of an effective stroke in one direction, brought about by doublets #2, #3 and #4, during a period when the central singlets are stiffened, and a recovery stroke, in the opposite direction, brought about by doublets #7, #8 and #9 while the central singlets relaxed. Doublet #1, and attached doublets #5 and #6, are essentially ineffective. It is the length of the axoneme that determines when the singlets are stiff and when relaxed, and when each lateral half doublet group is involved.

The most characteristic feature of ciliary motility is its biphasic nature, with the phases consisting of an effective stroke and a recovery stroke. The difference between these is, I believe, that the effective stroke involves the bending, at its base, of an essentially stiff organelle, while the recovery stroke involves the curvaceous return of a relaxed organelle. Any theory devised to account for ciliary movement must include, therefore, a mechanism for maintaining stiffness of the ciliary axoneme for half the duration of beat, and for achieving relaxation of this stiffness during the other half of the beat. As indicated above, there is evidence that the central singlets become stiff upon stimulation, and I have postulated a relationship between the length of the axoneme and the duration of stiffness of the central singlets for an "ideal" cilium. So, while the simplest possible way to achieve an effective stroke and a recovery stroke of equal duration might be if the axonemal length were exactly equal to the length involved in $\frac{1}{2}\lambda$ of beat, it seems obvious that variations from this "ideal" condition may also have evolved. There are, for instance, very short cilia—probably of a length much shorter than that involved in a half wavelength. But, in such a very short cilium, bending through any angle up to 180° for the effective stroke might be accomplished by fewer than all the doublets of the first lateral half of the axoneme. Stimulation of the remaining doublets of this lateral half could contribute nothing more to the effective stroke because the cilium is already parallel to the substratum. Yet the recovery stroke cannot begin until the first of the doublets of the other lateral half is stimulated to bend in the opposite direction. The central singlets need be stiff only for the period of actual movement of the effective stroke, and this duration of stiffness would be correlated with their length. This suggests that the motility of cilia with lengths less than that postulated for the "ideal" cilium could be equally adequately explained by this same general theory. Perhaps, therefore, in ciliary motility, one should speak of the effective stroke, together with its "rest" periods, as occupying the *time* for a half cycle rather than a half wavelength of beat, and the recovery stroke, and its "rest" periods, as occurring during the period of the second half cycle of beat.

In ciliary motility, I visualize the bending waves, as such, actually moving out ciliary doublets #7, #8 and #9, whereas only basal bending takes place in doublets #2, #3 and #4, with the waves beyond this basal bending point dampened completely by the stiffened singlets. Each bending wave initiated and moving out any one doublet advances a distance equivalent to the contour length involved in 1/9th wavelength in the time of 1/9th cycle. An equal distance is traversed in the next 1/9th cycle. Meanwhile, a new bending wave is initiated in the next doublet and follows the same pattern.

Flagellar movement

Planar motility of 9 + 2 flagella. The foregoing leads to the problem of planar flagellar motility, the directional role of the central singlet microtubules therein, and whether there are stops at doublet #1 and at doublets #5 and #6 to permit reversal of direction of what would otherwise be an essentially uniform helical beat. In 9 + 2 flagella, and in the variants of these (9 + 9 + 2) which also possess a pair of central singlets, the continuing axonemal length (at least as long as required for a full wavelength of beat and usually very much longer) leads to a considerably different situation from that existing for cilia. Because of the increased length, there is more viscous resistance of the medium to be overcome. However, the continuing propagation of the bending waves out the doublets, and of the stiffening wave out the singlets, provides a more stable set of conditions than exists in the simple cilium. We assume for flagella a stiffening wave in the central singlets, which, as in cilia, will extend ahead of the bending wave in-

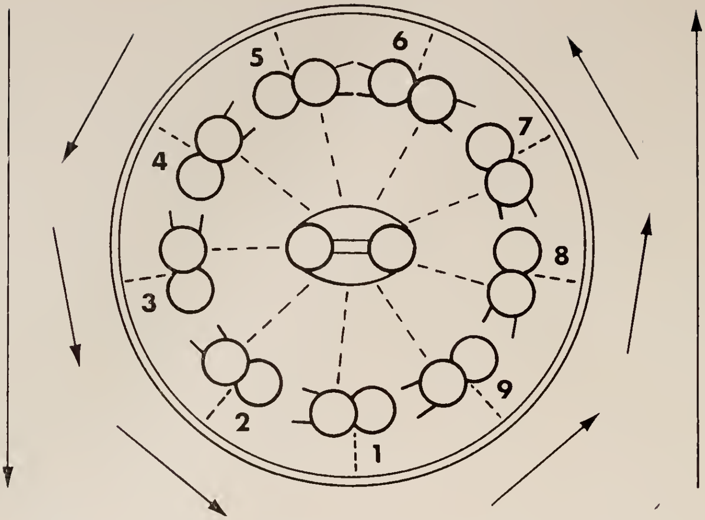


FIGURE 4. Diagram of cross-section of a 9 + 2 flagellum, showing direction of doublet bending for a planar wave.

augurated in the first doublet. The stiffness of the central singlets of a flagellum must persist in any given region for the duration of the active cycle of the wavelength.

As in cilia, basal activation of doublet #1 of the 9 + 2 flagellum will again be ineffective in producing bending, and for the same reasons. Activation of doublets #2, #3 and #4, successively, will result in the propagation of bending waves out the axoneme and produce its movement in one direction (Figs. 4 and 5). Resistance to lateral bending of doublets #5 and #6 is then encountered as they are activated, due to their attachment to each other, and to the continuing stiffness, *etc.*, of the central singlets. Now, however, because of the continuing length of the flagellar microtubules, the stiffening and bending waves do not terminate at the half-wavelength point, but continue moving out along the microtubules by which they are being propagated, for the full length of the axoneme. At the basal plate of the flagellar axoneme, 1/9 cycle after the activation of doublet #6, a bending wave is initiated in doublet #7, then successively in doublets #8 and #9, reversing the direction of the beat. With the singlets still stiff, these three doublets produce a planar return stroke. So, instead of an effective stroke and a recovery stroke, we have a complete planar wave propagated out the flagellum. The singlets may then relax, for the brief period between the activation of doublet #9, and the completion of the movement of the commutator around into position under doublet #1. Now, as the central singlets and doublets #1–#9, in order, are again successively activated at the base of the axoneme, a second wave, with the same characteristics, will be sent after the first. And, with the number present at any one time dependent upon the length of the axoneme, wave after wave will follow. With continuous flagellar motility, the distance apart of corresponding points of adjacent waves will be exactly one wavelength. The stiffened central singlets of the flagellum have three functions: (1) to decrease the amplitude of the waves, (2) to prevent or inhibit lateral bending, and (3) to provide resistance and thereby facilitate unbending. If there is a complete inhibition of lateral bending, so the axoneme bends first toward one end and then toward the other of the axis through doublet #1 which bisects the space between #5 and #6, the flagellar movement would be completely planar (*i.e.,* forward and back on Figs. 4 and 5).

Now, since the period of non-lateral movement at doublets #5 and #6, in preparation for the reversal of beat, is of approximately twice the duration of that at doublet #1, the more abrupt reversal at the latter might create a slight backlash, which would be repeated at one-wavelength intervals along the flagellum. This backlash might create the rotational force that causes the spermatozoon to turn slowly on its axis, as described by Gray (1958) and others for bull sperm.. There is, then, a slight asymmetry in this otherwise symmetrical planar beat.

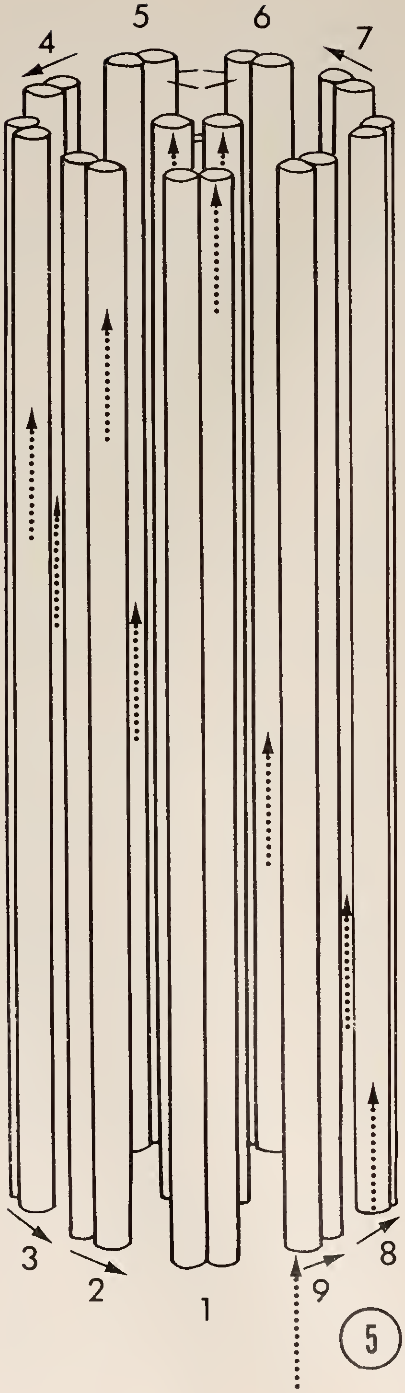
The complex bridges linking doublets #5 and #6 were described in the axoneme of echinoderm sperm tails by Afzelius (1959), in cilia of *Anodonta* by Gibbons (1961) and in gill cilia of *Elliptio* by Satir (1961). This bridge enables one to establish the axis of "bilaterality" which is correlated with the direction of the effective stroke of the cilium, which is related, also, to the orientation of the central singlets. It is not clear how widespread these #5–#6 bridges are in axonemes of spermatozoa. We have not seen them in any of the 9 + 0 or "9 + 1" axonemes of the acoels, rhabdocoels, triclads, or polyclads that we have examined. This suggests the possibility that they may be present only in cilia and in those sperm whose flagella have a planar beat.

The above view of planar flagellar motility is predicated on the supposition that the stiffening of the singlets provides sufficient resistance to lateral movement to inhibit completely actual bending of activated doublets #1, #5 and #6. Movement of the flagellum with bending at right angles to the plane through the centers of the two singlets must still be possible, however. Thus the bending of six doublets (#2, #3, #4 and #7, #8, #9), each in the direction away from its dynein arms, is responsible for this type of flagellar motility.

A less complete stiffening resistance to lateral bending may be the explanation for types of 9 + 2 axonemal motility intermediate between planar and helical. One must not ignore, however, the possible supplemental role in planar movement of components other than the singlets, present in some flagella, which are bilaterally disposed with respect to the plane of symmetry.

It is theoretically possible that under some circumstances, the activating stimuli to the central singlets of 9 + 2 flagellar axonemes are simply turned off or are non-functional. Under these circumstances these 9 + 2 axonemes would beat with a full helical beat.

9 + 0 axonemal motility. To account for axonemal motility of the 9 + 0 type. I suggest that a three-dimensional helical wave is generated by an impulse arising at the basal plate below doublet #1, and spreading around the plate in one direction, only, to produce propagated bending waves moving out each doublet, in serial order (Figs. 6, 7). Each doublet bends in a direction away from its dynein arms. If the commutator moves clockwise, the helical wave will be counterclockwise. A bending wave in any given doublet is 1/9 out of phase with the wave in either



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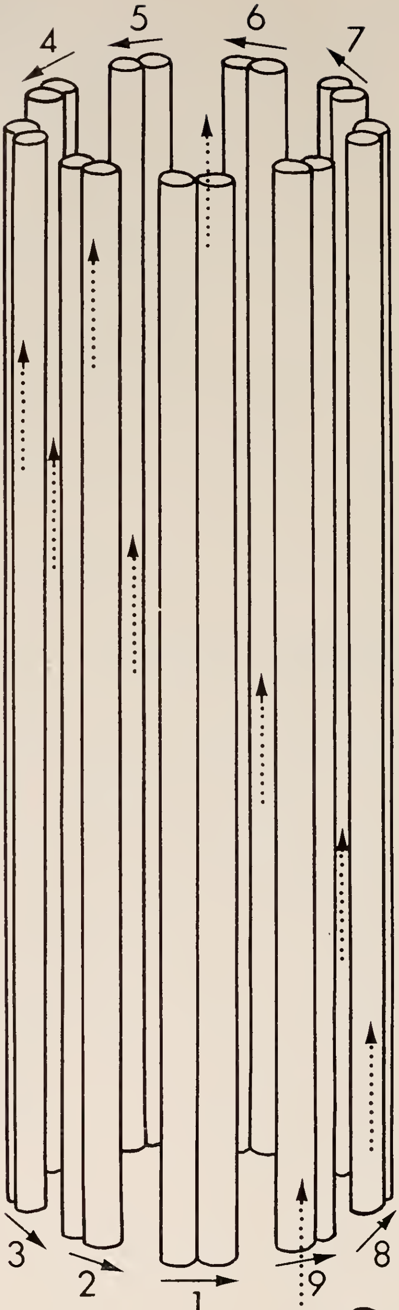

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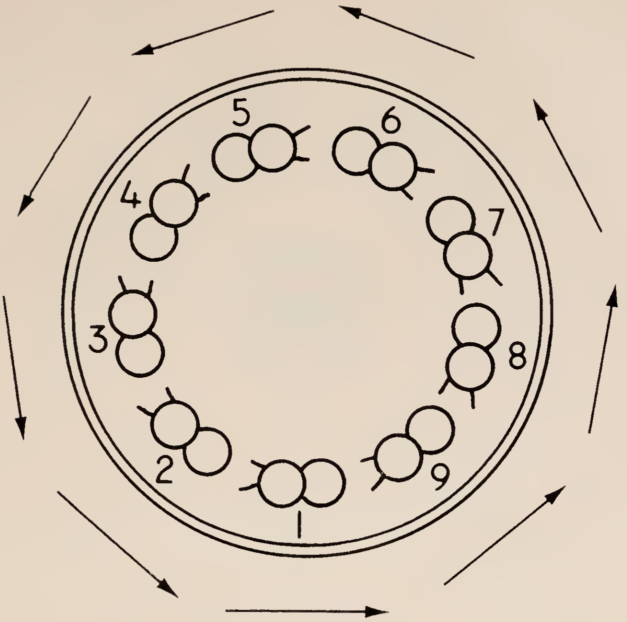

FIGURE 7. Diagram of cross-section of 9 + 0 flagellum, to indicate direction of successive bending of doublets to produce a wavelength of helical movement.

neighbor. Except for the directional involvement of the doublets (bending with the B-subtubule on the concave side) this view is essentially the same as that suggested by Bradfield (1955; pages 324–325) for the three-dimensional type of motility of 9 + 2 flagella (Fig. 9a). For these Bradfield included a statement that there was no conduction at all by the central fibrils. Of course, neither 9 + 0 nor "9 + 1" flagella had been discovered as early as 1955.

Logically, one wavelength of helical beat of a 9 + 0 sperm flagellum is produced by activation of doublets #1 through #9, and movement of the commutator back to #1 again, in preparation for the next wavelength of beat. As the first helical wave is propagated more distally along the flagellum, it is followed, without interruption, by successive waves of activation of doublets #1 through #9, the number of waves present at any one time depending on the length of the axoneme.

"9 + 1" axonemal motility. The "9 + 1" axoneme, with its 9 doublets and central core (Henley *et al.*, 1969) likewise moves with a helical wave (Fig. 8). It is assumed that the 9 doublets would be activated successively, in order, to produce one wavelength of helical beat. Successive waves would pass along the

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FIGURE 5. Diagram of side view of one wavelength of a 9 + 2 flagellum to show propagation of waves out the microtubules to give planar movement. The dynein arms have been omitted, with the exception of one pair on doublet #5.

FIGURE 6. Diagram of side view of one wavelength of a 9 + 0 flagellum, to show propagation of waves out the doublets to give helical movement. The dynein arms of the doublets have been omitted.

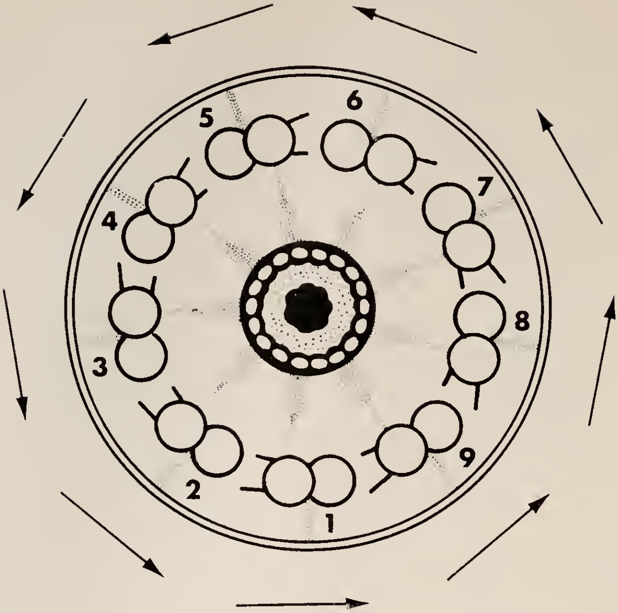

FIGURE 8. Diagram of a cross-section of a "9 + 1" flagellum to show central core and to indicate directions of successive bending of doublets to produce a wavelength of helical movement of reduced amplitude.

"9 + 1" axoneme, with the number present at any time depending upon axonemal length. Considerable resistance would be provided by the complex core because of its straightening tendencies (Henley, Costello, Thomas and Newton, 1969). However, since this resistance is nondirectional, as compared with that provided by a pair of central singlets, it may serve only to reduce the amplitude of beat, and perhaps contribute to unbending. It is of interest that some spermatozoa of triclad and polyclad flatworms have very long free flagella with "9 + 1" axonemes exceeding 200 microns in length, which are clearly observed to move with a helical beat.

DISCUSSION

Bradfield (1955) discussed possible mechanisms of flagellar and ciliary motility. He numbered the peripheral fibers 1 to 9, with #1 in the plane of symmetry. The direction of ciliary beat was assumed to be in the direction radially outward from fiber #1. For cilia, he assumed also that the impulse spreads around the ring in *both directions,* as well as to the central pair, which he considered to be more rapid conductors (Fig. 9a). For further details, see Bradfield's paper.

For flagella, Bradfield hypothesized that two-dimensional waves might be produced if the impulse spreads both directions around the base from #1 and dying out between #5 and #6 (or being reflected back) but *not* picked up and conducted rapidly along the flagellum by the central fibers in the manner postulated for the cilium. Bradfield suggested, also, that three-dimensional, corkscrew-like waves

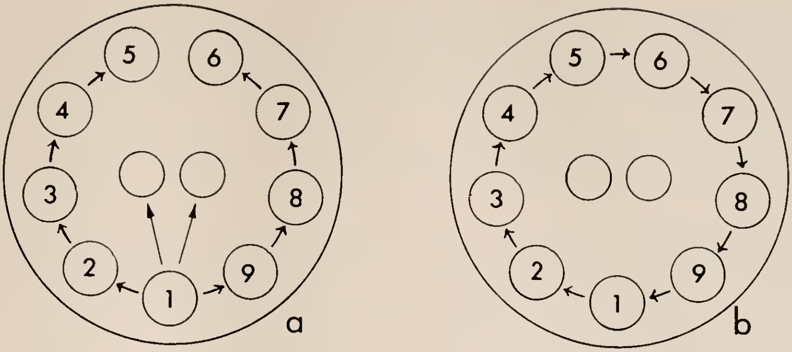

FIGURE 9. Bradfield's diagrams for (a) the ciliary beat and (b) the helical flagellar beat.

might be produced when the impulse originating under doublet #1 spread around the basal plate in one direction only, to fire off propagated contractions in each fiber in turn (Fig. 9b). Both these schemes for flagellar movement had the weakness of not suggesting any specific function for the central pair.

Minor modifications of Bradfield's theories by Gray (1955) and Sleigh (1960) present essentially the same difficulties. See also the reviews by Fawcett (1962), Sleigh (1962, 1968), Satir (1965b), and Holwill (1966, 1967).

Flagellar movement

There is a growing body of evidence from comparative studies by electron microscopy, suggesting that the central axonemal elements (whether they be the singlet microtubules of the 9 + 2 pattern or the core arrangement of "9 + 1" flagella of the sperm of certain flatworms) serve a stiffening function, acting in opposition to the bending of the doublet microtubules in effecting motility. The motile spermatozoa we have studied of those Turbellaria which have the 9 + 0 pattern (lacking any trace of central elements) have paired axonemes incorporated into two undulating membranes, each of which can move independently of the other, but which are normally integrated for effective motion. In some cases where the spermatozoa are very long and slender (notably the acoel flatworm *Polychoerus*) there is a keel-like central structure in the tail cytoplasm between the undulating membranes and their axonemes (Costello, unpublished data); this appears to be a centriole derivative, and to serve the stiffening function performed by the central elements of the axonemes in spermatozoa having such structures. The spermatozoon of the acoel *Childia* likewise has two 9 + 0 incorporated axonemes (Costello, Henley and Ault, 1969), but here there is no trace of the keel-like central structure. This may be correlated with the fact that the sperm of this form is much shorter and more compact than that of *Polychoerus* (50–60 microns *vs.* 400 microns in length) and the paired undulating membranes, into which the axonemes are incorporated, are considerably wider in the spermatozoon of *Childia*. Thus, the membranes themselves may impart the necessary opposing force of rigidity supplied by the central elements in sperm having other patterns of microtubule arrangement. The motile organelles of those flatworms with axonemes having the "9 + 1" arrangement (see Henley *et al.*, 1969, for references) may occur either as very long free flagella, as in *Dugesia* and *Bdelloura* (Silveira and Porter, 1964) and *Mesostoma* (Henley *et al.*, 1969) or as paired axonemes closely applied to the body of the spermatozoon (as in *Stylochus*) and connected to it by very fine filamentous material (Thomas, 1971). In either case, however, the core appears to be considerably more rigid than the doublets in negatively stained "9 + 1" preparations.

Since 9 + 2, "9 + 1", and 9 + 0 axonemes of spermatozoa of Turbellaria were all found to be motile (Costello *et al.*, 1969; Henley *et al.*, 1969; Bedini and Papi, 1970; Hendelberg, 1970), it is clear that the doublet microtubules (or some part of them—as, for example, the dynein arms) are the significant components responsible for motility of these axonemes. Motile sperm with 9 + 0 axonemes are known in other groups also (for references, see Afzelius, 1970; Phillips, 1969, 1970a, 1970b). True 9 + 1 (9 doublets and one singlet) axonemes have been described for spermatozoa of certain scorpions (Hood, Watson, Deason and Benton, 1972).

It is worthy of note that the axonemes of spermatozoa of lower invertebrates all lack the coarse dense outer fibers characteristic of the flagella of many mammalian sperm. This, again, points to the doublet microtubules as the "simple common denominator" of axonemal motility.

I consider it highly significant that the surface cilia of these Turbellaria are of a single type, namely, 9 + 2, regardless of whether the sperm axonemes are 9 + 0, "9 + 1," or absent altogether. This suggests that ciliary motility differs from flagellar motility in that the ciliary stroke always requires directional alignment and that the central singlets within the axoneme determine or contribute to this.

Brokaw (1965) gave the parameters for the bending waves of the flagella of *Lytechinus*, *Ciona* and *Chaetopterus* spermatozoa. He considered these flagellar waves to be planar (pages 156, 160). The duration of time for the completion of one wavelength of beat was approximately 1/30 second, as determined from their beat frequency. They showed wavelengths of 22.6, 22 and 19.5 microns, respectively, and the contour lengths of the waves as measured along the flagella were 29.6, 30 and 25.5 microns, respectively. In *Chaetopterus* sperm, there were 1.25 waves for the overall length of the flagellum (31.875 microns). The actual length of the flagellum involved depends upon the wavelength, amplitude and exact form of the wave, and can be calculated by use of a cited formula (page 158). This means that for the half wavelength, the distance measured along the flagellum is 14.8, 15 and 12.75 microns, respectively. The surface cilia of the turbellarians I have studied were 12 to 15 microns in length and thus fall precisely within this range.

Harris (1961) considered the possibility that the rigidity of the cilium is provided by the two central fibrils, the peripheral ones being entirely contractile, but rejected the idea on the basis of a theoretical calculation that led him to believe they would have to possess a modulus of elasticity as high as that of a steel wire. I am not convinced of the validity of Harris' calculations. The total resistance leading to rigidity of the main body of the cilium during the effective stroke involves considerably more than that supplied by the central singlets. The non-activated doublets, matrix, membrane, periodic connectives between doublets and central structures, connectives between doublets and membrane, nexin connectives, etc., must all offer some resistance both to propagated bending and to being bent backward by the medium. The stiffening supplied by the activated central singlets might be merely the final quantity that makes the effective stroke different from the recovery stroke.

Holwill (1966, page 750) has discussed this problem of compressive elements, involving these same components, and does not find impossible the likelihood of a high Young's modulus.

It is quite unnecessary to postulate, as Bradfield (1955), Sleigh (1962) and others have done, that turgor within the plasma membrane provides the stiffening. It was early shown that in glycerinated cilia, where the membranes and much of the matrix had been removed, the axonemes were still capable of movement. The recent experiments of Summers and Gibbons (1971) on isolated axonemes likewise show that turgor is not required.

Reversal of ciliary beat

In the very long (*ca.* 2000 microns) compound cilia of the comb-plates of *Mnemiopsis,* according to Afzelius (1961), the effective stroke is normally toward tubule #1, but in cilia of *Anodonta,* Gibbons (1961) has shown that the effective stroke is toward doublets #5 and #6. Since comb-plates of *Mnemiopsis* are capable of ciliary reversal and may, therefore, start their beat either toward doublet #1 or doublets #5 and #6, the question is, perhaps, academic.

However, in regard to reversal of direction of ciliary beat we may categorically state that if our view of the direction of bending of individual doublets is correct, reversal is not brought about by reversing the order of activation of the doublets. Instead, the reversed beat should start with activation at the opposite side (at doublets #5–6 instead of #1, for example), simultaneously sending impulses to the central singlets, and creating an effective stroke with doublets #7, #8 and #9, and the recovery stroke with doublets #2, #3 and #4. Hence the activating commutator would move in the same direction in the reversed and in the normal beat. In fact, if there were a brief refractory period after each ciliary stroke (to demarcate individual beats) there is no reason why the beat could not start with the activation of any one of the doublets. This might explain the various patterns of ciliary beating found by Sleigh (1968).

Ciliary versus flagellar motility

One of the significant facts emerging from these theoretical considerations is that a simple (non-compound) axonemal organelle, possessing stiffenable central singlets and of such length as to provide for only one-half wavelength or less must beat as a "typical" cilium, whereas a simple axonemal organelle of length equal to or greater than that needed to produce one wavelength at its characteristic amplitude and form would beat as a flagellum. The distinction between them is, therefore, length, in relation to wavelength, amplitude and form of beat. These constitute the basic differences between cilia and flagella.

If, under either experimental or "normal" conditions, the wavelength and amplitude relation of either type of organelle is sufficiently altered, a cilium might be induced to beat as a flagellum, or a flagellum as a cilium. The production of a helical wave ("flagellar" wave) in the cilia of *Paramecium multimicronucleatum* by Kuznicki, Jahn and Fonseca (1970), brought about by treatment of the animals with 1.0% methyl cellulose for 3 to 24 hours, is clearly an example of just such an alteration of beat. Brokaw (1965, page 160) records, "Bending waves of greatly reduced radii of curvature [and therefore shorter wavelength] were observed when *Chaetopterus* spermatozoa were suspended in seawater solutions to which methyl cellulose had been added to increase the viscosity." Solutions with viscosities up to 300 centipoises were used in his experiments. Tamm (1972) has confirmed the observations that an increase in the viscosity of the medium can alter the mode of beat.

It is freely acknowledged, as Kuznicki *et al.* (1970) have pointed out, that all cilia, under all circumstances, do not beat with the back-and-forth movement ordinarily considered typical of these organelles. Similarly, all flagella, under all circumstances, do not beat with the helical undulations that are so much a part of all classical descriptions of flagellar motility. Students of protozoology and spermatology are well aware of the infinite variety of types of movement of these axonemal organelles. However, this theory is being presented as a simple basic view of axonemal mechanics and makes no attempt to explain the special features of unusual variations of motility other than 9 + 0 and "9 + 1" (9 + core) helical movement.

Sliding and bending mechanisms in motility of cilia and flagella

Summers and Gibbons (1971) presented evidence in favor of a sliding filament theory, with "ATP-induced shearing forces between outer tubules which, when resisted by the native structure, lead to localized sliding and generate an active bending movement" (page 3092). Isolated flagellar axonemes, briefly digested with trypsin, were found to actively disintegrate into individual tubules and groups on the addition of ATP. The disintegration resulted from active sliding between groups of doublets, together with a tendency for the partially disintegrated axoneme to coil into a helix (Summers and Gibbons, 1971).

The present paper places emphasis upon the bending activities which occur within the individual doublets and suggests that these may be of primary importance in the basic mechanism by which motility of cilia and flagella is accomplished. In normal motility, if the doublets remain attached to the basal plate, and if there is no change in their length (as observed for cilia by Satir, 1965a), the apparent sliding at the distal ends of the tubules might be incidental to whatever bending is taking place. Very little sliding movement appears to be possible in the intact cilium or flagellum, where the several types of connectives described above hold the component parts in relatively constant positions with respect to each other. Cilia or flagella which are just sufficiently macerated (with PTA) to destroy the connectives between doublets but not their attachments to the basal plate show the doublets splayed out in particular directions, bent, but not crawling over each other. In such isolated, partially macerated cilia, it has been possible in some cases to identify the doublets by number (Figs. 7 and 8 of the first paper of this series, Costello, 1973) and the directions of their bending correlated with their positions of attachment. Measurements of these doublets after moderate PTA maceration show no increase in length as compared with those in an intact cilium. In addition, individual doublets can be isolated from their basal plates and will still show bending activities. However, our attention has been concentrated on the bending, looping, coiling, and spiraling activities of the doublet microtubules, since there was no direct evidence in this work to indicate that active sliding of doublets relative to each other was taking place. The information presented does not enable one to evaluate the relative merits of doublet interaction due to a sliding mechanism involving the dynein arms versus conformational changes in the subunit arrangements within the doublet microtubules themselves. Possibly both factors eventually may be shown to be involved.

Instead, the observations have been collated in an attempt to suggest the simple mechanics whereby a propagated unidirectional bending force acting in successive doublets, combined with stiffening activities in the central singlets (of 9 + 2 organelles) or central core (of "9 + 1" flagella), could give rise to either planar or helical movements of an axoneme, and, by interaction involving other features of the organelles, account for both ciliary and flagellar motility of various types.

Mechanisms for axonemal and microtubular motility may have evolved along pathways far different from those followed by mechanisms for contraction of striated muscle.

I am greatly indebted to Drs. Catherine Henley, Shinya Inoué and Lewis Tilney for reading the manuscript and making many valuable suggestions for its improvement. Sincere appreciation to Dr. Henley for taking practically all the electron micrographs upon which the supporting observations were based is here recorded, also.

SUMMARY

1. A simple basic theory to account for the mechanics of ciliary and flagellar motility is proposed. It is based in large part on the conclusions resulting from observations made on axonemes of surface body cilia and of sperm flagella of a number of lower invertebrates after maceration and negative staining with phosphotungstate.

2. Ciliary motility consists of an effective stroke brought about by the successive bending of the three doublet microtubules of one lateral half of the axoneme while the central singlet microtubules are stiffened, and a recovery stroke, similarly brought about by the three doublets of the other lateral half, while the central singlets are relaxed. Bending of each doublet is in the direction away from its dynein arms. Doublet #1, and attached doublets #5 and #6, are rendered essentially ineffective by the stiffened singlets. The length of the axoneme in relation to wavelength determines when the singlets are stiff and when relaxed, and when each lateral half doublet group is involved.

3. Planar motility (of 9 + 2 flagella) consists of movement in one direction, brought about by successive bending of the three doublets of one lateral half of the axoneme, followed by movement in the other direction due to bending of the three doublets of the other lateral half, while the central singlets remain stiffened for the active period of the entire wavelength. Movement of doublets #1, #5, #6 is

inhibited by the stiffened singlets. It is the greater length of the flagellum that is chiefly responsible for the difference between flagellar and ciliary motility.

4. The helical motility of 9 + 0 and "9 + 1" axonemes is brought about by successive bending waves moving out all nine doublet microtubules, in sequential order, with no directional inhibition of any of them. The stiffened core of the "9 + 1" axoneme would be expected to decrease the amplitude of the helical beating in the latter type.

LITERATURE CITED

Afzelius, B., 1959. Electron microscopy of the sperm tail: results obtained with a new fixative. *J. Biophys. Biochem. Cytol.*, **5**: 269–278.

Afzelius, B. A., 1961. Some problems of ciliary structure and ciliary function. Pages 557–567 in T. W. Goodwin and O. Lindberg, Eds., *Biological Structure and Function*, vol. II. Academic Press, London, New York.

Afzelius, B. A., 1970. Thoughts on comparative spermatology. Pages 565–573 in B. Baccetti, Ed., *Comparative Spermatology*. Academic Press, New York, London.

Bedini, C., and F. Papi, 1970. Peculiar patterns of microtubular organization in spermatozoa of lower Turbellaria. Pages 363–366 in B. Baccetti, Ed., *Comparative Spermatology*. Academic Press, New York, London.

Bradfield, J. R. G., 1955. Fibre patterns in animal flagella and cilia. *Symp. Soc. Exp. Biol.*, **9**: 306–334.

Brokaw, C. J., 1965. Non-sinusoidal bending waves of sperm flagella. *J. Exp. Biol.*, **43**: 155–169.

Brokaw, C. J., 1968. Mechanisms of sperm movement. *Symp. Soc. Exp. Biol.*, **22**: 101–116.

Brokaw, C. J., S. F. Goldstein and R. L. Miller, 1970. Recent studies on the motility of spermatozoa from some marine invertebrates. Pages 475–486 in B. Baccetti, Ed., *Comparative Spermatology*. Academic Press, New York, London.

Costello, Donald P., 1973. A new theory on the mechanics of ciliary and flagellar motility. I. Supporting observations. *Biol. Bull.*, **143**: 279–291.

Costello, D. P., C. Henley and C. R. Ault, 1969. Microtubules in spermatozoa of *Childia* (Turbellaria, Acoela) revealed by negative staining. *Science*, **163**: 678–679.

Fawcett, D. W., 1962. Sperm tail structure in relation to the mechanism of movement. Pages 147–169 in D. W. Bishop, Ed., *Spermatozoan Motility*. American Association Advancement of Science, Washington, D. C.

Gibbons, I. R., 1961. The relationship between the fine structure and direction of beat in gill cilia of a lamellibranch mollusc. *J. Biophys. Biochem. Cytol.*, **11**: 179–205.

Gray, J., 1955. The movement of sea urchin spermatozoa. *J. Exp. Biol.*, **32**: 775–801.

Gray, J., 1958. The movement of the spermatozoa of the bull. *J. Exp. Biol.*, **35**: 96–108.

Harris, J. E., 1961. The mechanics of ciliary movement. Pages 22–36 in J. A. Ramsay and V. B. Wigglesworth, Eds., *The Cell and the Organism*. Cambridge University Press, Cambridge.

Hendelberg, J., 1970. On the number and ultrastructure of the flagella of flatworm spermatozoa. Pages 367–374 in B. Baccetti. Ed., *Comparative Spermatology*. Academic Press, New York, London.

Henley, C., D. P. Costello, M. B. Thomaš and W. D. Newton, 1969. The "9 + 1" pattern of microtubules in spermatozoa of *Mesostoma* (Platyhelminthes, Turbellaria). *Proc. Nat. Acad. Sci. U. S.*, **64**: 849–856.

Holwill, M. E. J., 1966. Physical aspects of flagellar movement. *Physiol. Rev.*, **46**: 696–785.

Holwill, M. E. J., 1967. Contractile mechanisms in cilia and flagella. Pages 287–333 in D. R. Sanadi, Ed., *Current Topics in Bioenergetics*, vol. 2. Academic Press, New York.

Hood, R. D., O. F. Watson, T. R. Deason and C. L. B. Benton, Jr., 1972. Ultrastructure of scorpion spermatozoa with atypical axonemes. *Cytobios*, **5**: 167–177.

Kuznicki, L., T. L. Jahn and R. J. Fonseca, 1970. Helical nature of the ciliary beat of *Paramecium multimicronucleatum*. *J. Protozool.*, **17**: 16–24.

Parducz, B., 1953. Zur Mechanik der Zilienbewegung. *Acta Biologica Acad. Sci. Hungaricae*, **4**: 177–220.

PARDUCZ, B., 1954. Reizphysiologische Untersuchungen an Ziliaten. II. Neuere Beiträge zum Mechanismus der Ziliatur. *Acta Biologica Acad. Sci. Hungaricae,* **5**: 169–212.

PARDUCZ, B., 1961. Cilia. Pages 232–233 in P. Gray, Ed., *The Encyclopedia of the Biological Sciences.* Reinhold Publishing Corp., New York.

PARDUCZ, B., 1967. Ciliary movement and coordination in ciliates. *Int. Rev. Cytol.,* **21**: 91–128.

PHILLIPS, D. M., 1969. Exceptions to prevailing patterns of tubules (9 + 9 + 2) in the sperm flagella of certain insect species. *J. Cell. Biol.,* **40**: 28–43.

PHILLIPS, D. M., 1970a. Insect sperm: their structure and morphogenesis. *J. Cell Biol.,* **44**: 243–277.

PHILLIPS, D. M., 1970b. Insect flagellar tubule patterns. Theme and variations. Pages 263–273 in B. Baccetti, Ed., *Comparative Spermatology.* Academic Press, New York, London.

SATIR, P. G., 1961. The mechanism of ciliary motion: a correlation of morphological and physiological aspects of ciliary beat of the gill epithelium of the freshwater mussel, *Elliptio complanatus* (Solander). *Ph.D. dissertation, Rockefeller University.*

SATIR, P., 1965a. Studies on cilia. II. Examination of the distal region of the ciliary shaft and the role of the filaments in motility. *J. Cell Biol.,* **26**: 805–834.

SATIR, P., 1965b. Structure and function in cilia and flagella. *Protoplasmatologia,* **3E**: 1–52.

SILVEIRA, M., AND K. R. PORTER, 1964. The spermatozoids of flatworms and their microtubular systems. *Protoplasma,* **59**: 240–265.

SLEIGH, M., 1960. The form of the beat of cilia of *Stentor* and *Opalina. J. Exp. Biol.,* **37**: 1–10.

SLEIGH, M. A., 1962. *The Biology of Cilia and Flagella.* Pergamon Press, Oxford.

SLEIGH, M. A., 1968. Patterns of ciliary beating. *Symp. Soc. Exp. Biol.,* **22**: 131–150.

STEPHENS, R. E., 1970. Isolation of nexin—the linkage protein responsible for maintenance of the nine-fold configuration of flagellar axonemes. *Biol. Bull.,* **139**: 438.

SUMMERS, K. E., AND I. R. GIBBONS, 1971. Adenosine triphosphate-induced sliding of tubules in trypsin-treated flagella of sea urchin sperm. *Proc. Nat. Acad. Sci. U. S.,* **68**: 3092–3096.

TAMM, S. L., 1972. Ciliary motion in *Paramecium.* A scanning electron microscope study. *J. Cell. Biol.,* **55**: 250–255.

THOMAS, M. B., 1971. A fine-structural analysis of the spermatozoa of *Stylochus zebra* (Turbellaria, Polycladida). *Ph.D. dissertation, University of North Carolina.*

Reference: *Biol. Bull.*, **145**: 310–322. (October, 1973)

THE INTERMOLT CYCLE OF CIRRIPEDS: CRITERIA FOR ITS STAGES AND ITS DURATION IN *BALANUS AMPHITRITE*

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Many physiological and behavioral responses in crustaceans change markedly during the course of an intermolt cycle. To take such variations into account it is necessary to identify the stages of the intermolt cycle. Drach (1939, 1944) successfully divided the intermolt cycle of brachyurans and natantians into four main stages, A, B, C, and D, and, using structural changes in the integument, developed criteria by which these stages could be identified. Drach's method has found wide application within the Malacostraca (see Yamaoka and Scheer, 1970). In the Cirripedia, however, an intermolt staging method for live animals chosen at random has not been available. Studies which have related to the intermolt cycle in cirripeds have generally used animals at timed intervals after ecdysis (Patel and Crisp, 1961; Barnes and Barnes, 1963; Costlow, 1963; Shimony and Nigrelli, 1972). A requisite for the successful application of the timed interval method is intermolt cycles of uniform duration. This condition, however, may not be met in the cirripeds. Costlow and Bookhout (1953, 1956) found wide variations in the durations of the intermolt cycles of juvenile specimens of *Balanus improvisus* and *B. amphitrite*; there is no information in this regard for adult barnacles.

Intermolt stages in cirripeds have been morphologically identified in only a few studies. Using the criteria of Drach, Bocquet (1956) identified Stages D_1 and D_2 in *Chthamalus stellatus* by microscopically examining the cirri. Bocquet-Vedrine (1965) developed a method for histologically identifying intermolt stages of *Elminius modestus* in thin sections of whole animals. The present study had two major objectives: (1) to develop an intermolt staging method for barnacles which can utilize live, intact specimens taken at random, and (2) to statistically analyze the intermolt cycle of adult specimens of *B. amphitrite*.

MATERIALS AND METHODS

Specimens of the intertidal barnacle *Balanus amphitrite amphitrite* Darwin (Harding, 1962) were collected from the noncreosoted portions of the laboratory dock in Beaufort, North Carolina, from February to November, 1972. The rostro-carinal diameter of the animals ranged from 5 to 10 mm. The animals were maintained in aquaria containing continuously aerated seawater at a salinity of 30‰ and fed *Artemia* nauplii (Metaframe San Francisco Bay Brand) for one month prior to experimentation. During the experiments the animals were maintained individually in compartmentalized plastic boxes in 40 to 50 ml of seawater at a salinity of 30‰. The boxes were kept in culture cabinets at 23° C with a LD 12:12 photoperiod. The seawater was changed and the animals fed 24 hr old *Artemia* nauplii daily. The compartments were checked for exuviae twice daily. Ecdysis was defined as having occurred midway between the observation when an exuvium was discovered and the previous observation. Animals which produced a brood during the experimental period, or which had ovigerous lamellae at the end of the period were discarded.

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For the development of criteria for the intermolt stages rami were snipped off of each animal successively during the course of one to three intermolt cycles at approximately one day intervals. Rami were obtained with a pair of fine forceps, snipping while the animals were pumping or feeding. Only rami from the 4th, 5th, and 6th cirri were used. For microscopic examination the rami were mounted on slides in either seawater or glycerin jelly. For the latter method a number of slides were prepared beforehand by placing a drop of molten glycerin jelly on each slide and allowing it to solidify. The rami were placed in a drop of seawater atop the glycerin jelly, a coverslip was added, and the slide was placed on a slide warmer (48 to 50° C) just long enough to liquefy the glycerin jelly. It was necessary to examine the rami in both seawater and glycerin jelly. In seawater the ramial tissues have high contrast and are easily seen, however, it is difficult to resolve the exoskeletal layers because of extensive refraction. Glycerin jelly reduces the amount of refraction and thus improves the resolution of the exoskeleton, but at the same time obscures the other ramial structures. Observations and measurements of the exoskeletal layers were accordingly made on glycerin jelly mounted rami while observations of the other ramial structures were made on seawater mounted rami.

Measurements of the exoskeletal layers were made with a filar occular micrometer (American Optical) in the middle portion of the posterior side of the segment. Unless otherwise stated all measurements were made on the 13th segment from the tip of the ramus. Repeated measurements showed that the method was reproducible within ±5 per cent. The accuracy of the measurements and the visible presence of the exoskeletal layers in the whole mounts were checked further by making parallel measurements on whole mounted rami and sectioned rami: The bodies of eight animals were dissected free of their shells and one ramus from each was mounted in glycerin jelly. The remainder of each of the bodies was fixed in Helly's fluid, dehydrated in a graded series of ethanol, cleared in xylene, and embedded in *Paraplast*. Serial sections were cut at 7 to 8 μ and stained according to Hubschman's (1962) modification of Gomori's Azan method. In the whole mounts the posterior-anterior diameter and the thickness of the procuticle and of the exocuticle were measured. In the sections the thicknesses of the procuticle and exocuticle were measured at a point in a ramus where the diameter was the same as that in the corresponding whole mounted ramus. The measurements of the whole mounts agreed well with those of the sectioned material: The correlation coefficients for the measurements of the procuticle and exocuticle were 0.85 and 0.97, respectively.

Microscopic examinations and photomicrographs were made with a Leitz Labolux microscope. Statistical analyses were made according to Sokal and Rohlf (1969).

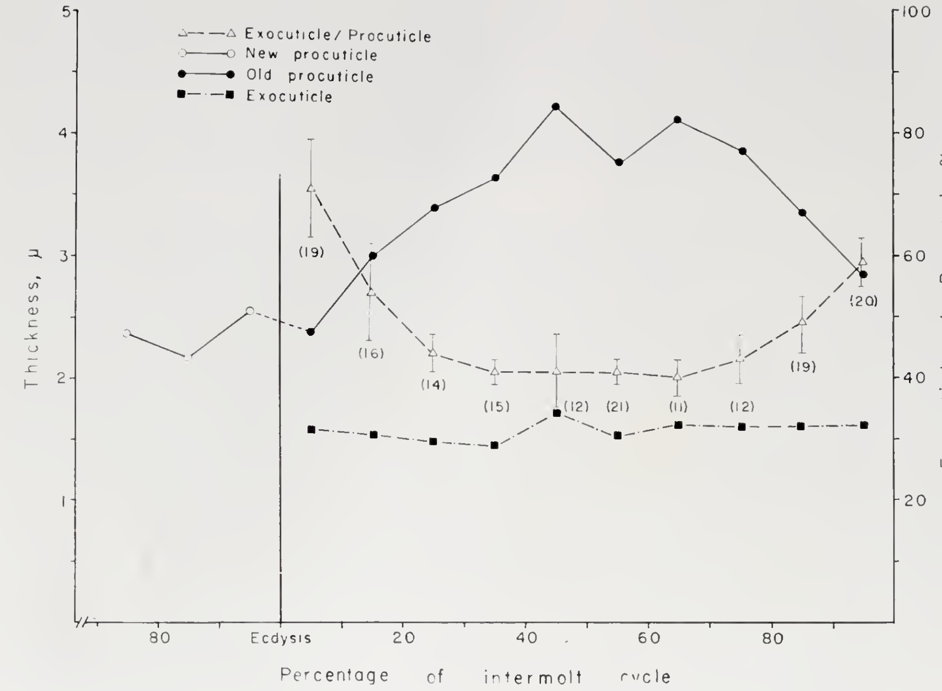


FIGURE 1. Thickness of the procuticle and of the exocuticle of *Balanus amphitrite* during the intermolt cycle. The stippled line shows the exocuticle expressed as a percentage of the corresponding procuticle. Each point is the mean of observations (number in parentheses) made for each 10 per cent interval of the intermolt cycle. The vertical bars represent 95% confidence limits.

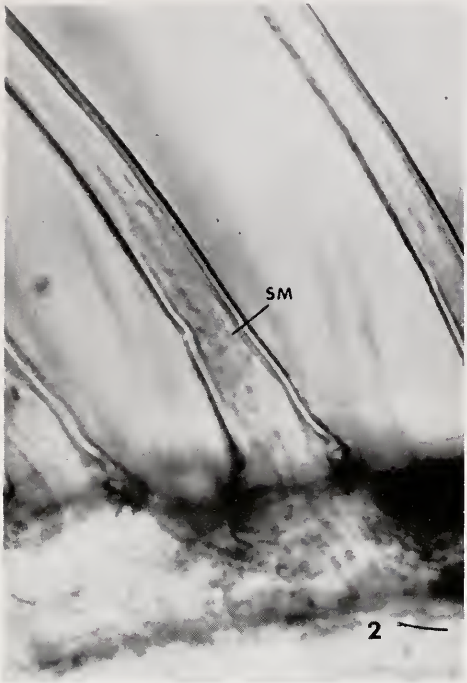
RESULTS

Criteria for the intermolt stages

Following the general outline of Drach (1939) we have divided the intermolt cycle of *B. amphitrite* into eight stages: A; B_1 and B_2; C; D_0, D_1, D_2, and D_3. The criteria by which these stages are identified are based upon (1) the overall form of the cirri, (2) the formation and the relative thicknesses of the exoskeletal layers, and (3) the progression of setogenesis.

The cirri. In a barnacle at rest the cirri normally assume a tightly curled form. Only in the first hours of postecdysis do the cirri deviate from this form and are tortuous, or twisted.

The exoskeleton. The exoskeleton of the rami as seen in the light microscope is composed of two layers (see Fig. 6). In live whole mounted rami and in glycerin jelly mounted rami the outer layer is dark green in color and the inner layer is light green. In sections of rami stained with Azan the outer layer is red and the inner layer blue. The outer and inner layers are assumed to be homologous (see Discussion) with the exocuticle and endocuticle, respectively, of the procuticle (the procuticle = exocuticle + endocuticle) of other crustaceans and the arthro-



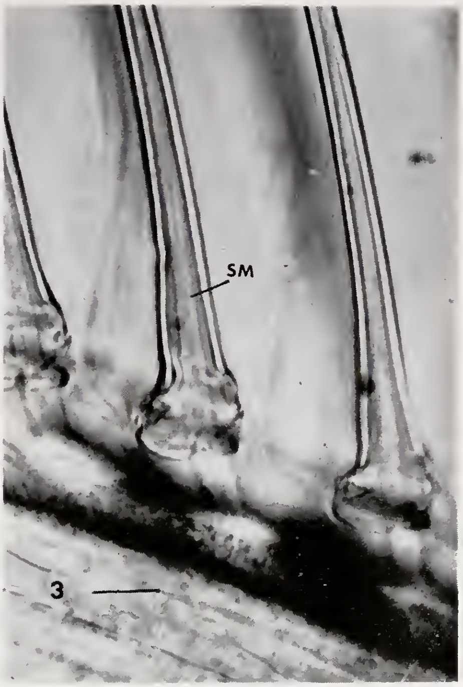


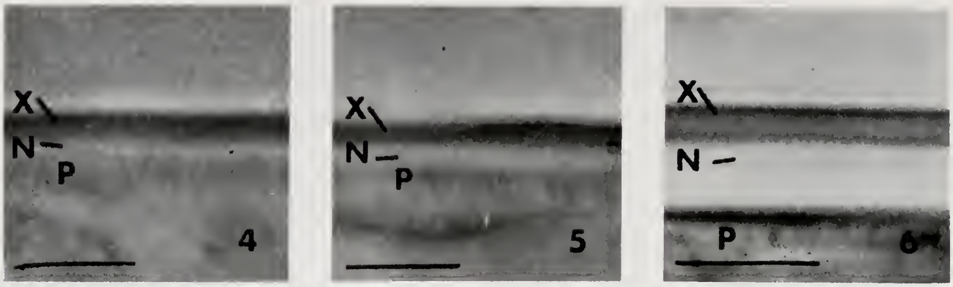
FIGURE 2. Setae of *Balanus amphitrite* in Stage C showing the setal matrix (SM) in the expanded state; ramus mounted in seawater; marker = 10 μ.

FIGURE 3. Setae of *Balanus amphitrite* in Stage D_0 showing the setal matrix (SM) in the contracted state; ramus mounted in seawater; marker = 10 μ.

pods in general (Hackman, 1971). These terms will be used throughout this paper.

As shown in Figure 1 a new procuticle is laid down in proecdysis before the animal has passed through 75 per cent of the intermolt cycle. The first presence of the new procuticle is difficult to distinguish from the granular epidermis. We have defined the new procuticle to be present when the segmental hinge on the posterior side of the ramus is present and when the layer making up the hinge is clear and nongranular in appearance (see Fig. 7). The new procuticle and the same procuticle immediately after ecdysis has a light green appearance and cannot be resolved into its component layers. The thickness of this procuticle, however, is greater than the thickness of the exocuticle (Fig. 1). We interpret this as meaning that the procuticle in this state is composed of the exocuticle and a small portion of the endocuticle but that they are not visibly differentiated, *i.e.*, the exocuticle is not sclerotized.

Within four hours after ecdysis the procuticle can be resolved into an exo- and endocuticle. The exocuticle then remains constant in thickness throughout the intermolt cycle while the procuticle increases in thickness in the first portion of the intermolt cycle and decreases in the last portion (Fig. 1). The changes in thickness of the procuticle thus reflect the changes which are occurring in the endo-



FIGURES 4–6. The exoskeleton on the posterior side of rami of *Balanus amphitrite* in post- and interecdysial stages: Figure 4 = Stage B_1; Figure 5 = Stage B_2; Figure 6 = Stage C; rami mounted in seawater; X = exocuticle, N = endocuticle, P = epidermis; markers = 5 μ.

cuticle. The stippled line in Figure 1 shows the thickness of the exocuticle expressed as a percentage of the thickness of the corresponding procuticle. This exocuticle percentage decreases from a high mean value of about 70 per cent in the first 10 per cent of the intermolt cycle to a mean of about 40 per cent between 30 and 70 per cent of the cycle, and then increases to close to a mean of 60 per cent in the last 10 per cent of the cycle. We have used the exocuticle percentage to define the limits of various intermolt stages since the values are indications as to whether the endocuticle is in a state of formation, steady state, or resorption.

Setogenesis. The setae of the rami are filled with a tissue matrix, the setal matrix. This matrix is continuous with a fibrous strand of tissue, the extrasetal matrix, which traverses the segment diagonally in the hypodermis. In post- and interecdysis the setal matrix is in an expanded state, *i.e.*, it completely fills the setae and has a loose appearance (Fig. 2). The first sign of proecdysis is a contraction of the setal matrix whereby a separation is formed between the setal exoskeleton and the setal matrix which now has a fibrous appearance (Fig. 3). A separation between the epidermis and the exoskeleton on the anterior side of the segment may also be found at this time.

Setogenesis then proceeds and the new setae are formed as invaginations running diagonally in the segment following the path of the extrasetal matrix (see Fig. 8). The invaginations are initiated anteriorly and proceed posteriorly in the segment. A complete invagination of the large, distal setae of a segment may run completely through its own segment and enter the next proximal segment. The tip of the new seta is formed within the basal portion of the old seta.

Criteria for the identification of the intermolt stages of *B. amphitrite* are then as follows:

Stage A (early postecdysis). The cirri are tortuous and the procuticle is thin and seemingly single-layered. There is no feeding activity or cirral beating but slow irregular movements of the cirri and opercular valves may be observed.

Stage B (late postecdysis). The cirri have regained their normal curled form. The setal matrix is expanded (Fig. 2). The procuticle is double-layered and the exocuticle percentage is more than 50 per cent. Stage B_1 is characterized by an exocuticle percentage of more than 65 per cent (Fig. 4) and Stage B_2 is characterized by an exocuticle percentage between 50 and 65 per cent (Fig. 5).

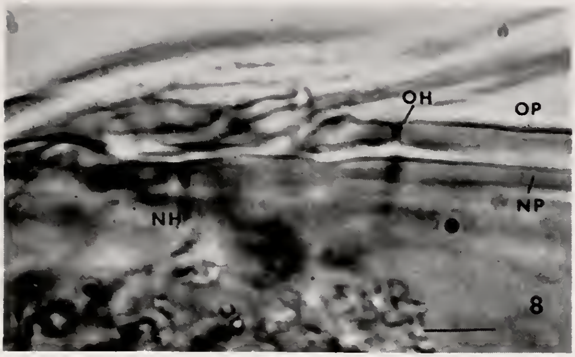


FIGURE 7. Posterior side of a ramus of *Balanus amphitrite* in Stage D_2 showing the new (NP) and old (OP) procuticles and the new (NH) and old (OH) segmental hinges; ramus mounted in glycerin jelly; marker = 5 μ.

Stage C (intercedysis). The setal matrix is expanded and the exocuticle percentage is 45 per cent or less (Fig. 6).

Stage D (proecdysis). Stage D_0 is characterized by the contraction of the setal matrix (Fig. 3), the rami otherwise having the appearance of Stage C. Stage D_1 is characterized by the presence of invaginations. During this stage the setal matrix retracts and the tip of the new seta is formed. Stage D_1 may be subdivided into two or more stages, the subdivisions being based on the increasing depth of the invaginations. Stage D_2 is characterized by the presence of a new procuticle on the posterior side of the segment (Fig. 7). The invaginated setae are more distinct and setules can be observed clearly on the tip of the new setae and faintly on the invaginated portion. The exocuticle percentage increases but is less than 60 per cent. Stage D_3 is characterized by an exocuticle percentage above

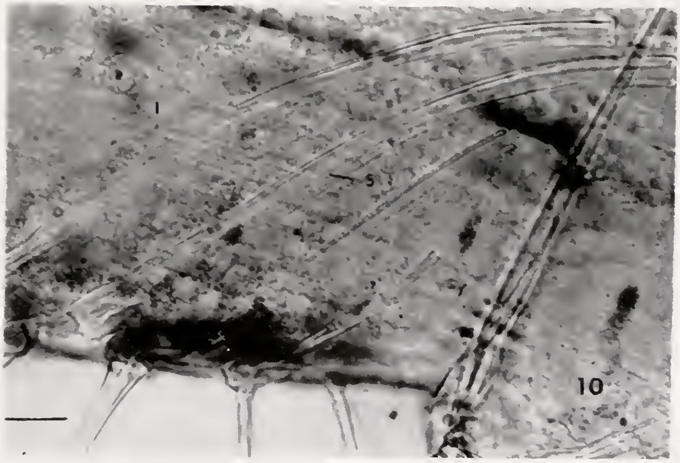


FIGURE 8. Invaginations (I) of the new setae of *Balanus amphitrite* in Stage D_3; ramus mounted in seawater; S = setules; marker = 100 μ.

TABLE I

Criteria for the intermolt stages of Balanus amphitrite

Intermolt stage	Cirri	Exocuticle percentage**	Setal matrix*	Invagination*	New procuticle**
A	Tortuous	100***	Expanded	Absent	Absent
B					
B_1	Normal	>65	Expanded	Absent	Absent
B_2	Normal	50–65	Expanded	Absent	Absent
C	Normal	<45	Expanded	Absent	Absent
D					
D_0	Normal	<45	Contracted	Absent	Absent
D_1	Normal	<60	Retraction or new tip	Faint	Absent
D_2	Normal	<60	New tip	Distinct	Present
D_3	Normal	>60	New tip	Obvious	Present

* Observed in seawater mounted rami.
** Observed in glycerin jelly mounted rami.
*** Seemingly single-layered.

60 per cent. The new procuticle is clearly seen and is thicker than the old exocuticle. The invaginated setae stand out brownish-yellow to the surrounding tissues (Fig. 8). Setules are clearly seen both on the tip and on the invaginated portion of the new setae.

A summarized outline of the intermolt stages and their criteria is presented in Table I.

Inter- and intraramial synchrony

The following studies were performed to determine whether the integumental changes in different segments of a ramus and the different rami of an animal proceed at an equal rate.

TABLE II

Thickness (μ) of the exocuticle (Exo) and procuticle (Pro) in segments of the inner (i) and outer (o) rami of the right 4th, 5th, and 6th cirri of a Stage C specimen of Balanus amphitrite. *The thickness of the exocuticle is also expressed as a percentage (Exo %) of the corresponding procuticle*

Ramus	5th Segment			13th Segment			20th Segment			Exo % mean ± S.E.
	Exo	Pro	Exo %	Exo	Pro	Exo %	Exo	Pro	Exo %	
6i	0.8	2.4	33	1.7	4.4	39	2.1	5.9	36	36 ± 1.4
6o	0.9	2.6	35	1.7	5.1	33	2.3	5.8	40	36 ± 1.4
5i	1.1	2.7	41	1.8	4.9	37	2.2	6.5	34	37 ± 1.7
5o	1.0	2.8	36	1.7	5.1	33	2.0	6.1	33	34 ± 0.8
4i	0.9	2.7	33	1.7	5.5	31	2.2	6.3	35	33 ± 0.9
4o	1.0	2.6	38	1.7	4.8	35	2.4	6.4	38	37 ± 0.8
Mean ± S.E.	0.95 ± 0.04	2.63 ± 0.05	36 ± 1.1	1.72 ± 0.02	4.97 ± 0.13	35 ± 1.1	2.20 ± 0.05	6.20 ± 0.11	36 ± 1.0	

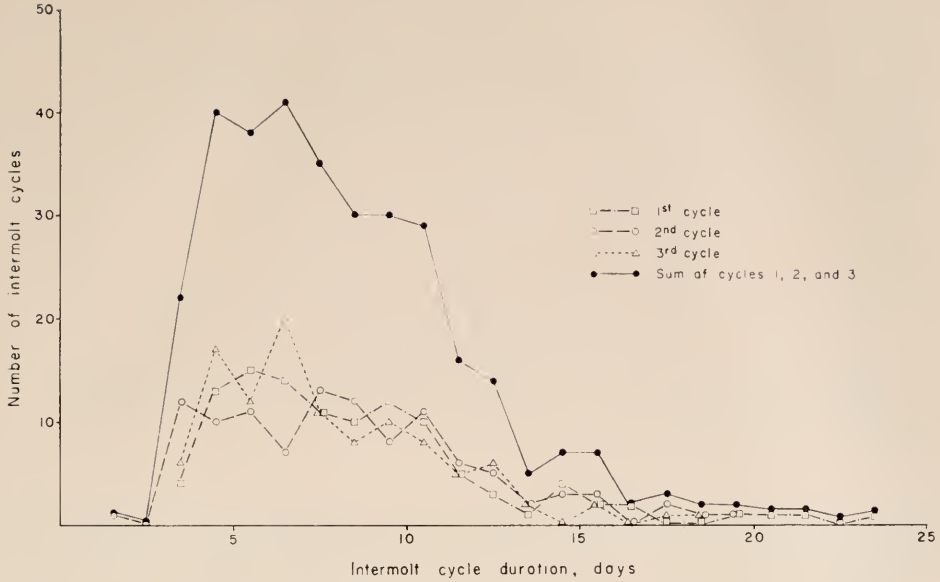


FIGURE 9. Frequency distribution of the durations of intermolt cycles of adult *Balanus amphitrite* kept in the laboratory at 23° C through three successive cycles.

The 5th, 13th, and 20th segments of the inner and outer rami of the right cirri of an animal were examined for variations in the thicknesses of the exoskeletal layers. The results (Table II) show that the exocuticle and the procuticle increase in thickness proximally within a ramus while the exocuticle percentages seem to be equal. The exocuticle percentages between the rami also seem to be equal.

The exocuticle percentages were calculated for the 13th segment of the inner rami of the right cirri of animals in different intermolt stages. Table III shows that the exocuticle percentages in a given animal had only small variations from ramus to ramus. Using the criteria in Table I this variation would not lead to different intermolt stages.

The integumental changes were characterized in the 5th, 13th, and 20th segments of the inner rami of the right cirri in nine animals in Stage D: two in D_0, three in D_1, three in D_2, and one in D_3. No differences were observed in the integumental changes either within or between the rami.

The above results show that setogenesis and the formation and resorption of the exoskeletal layers progress synchronously both inter- and intraramially.

Duration of the intermolt stages

The durations of the intermolt stages were calculated from the data derived from the animals used in developing the above criteria. The intermolt cycles of these animals ranged from 3 to 29 days (see also description of duration of intermolt cycle). Stage A was found to last less than four hours. Stage B lasted from

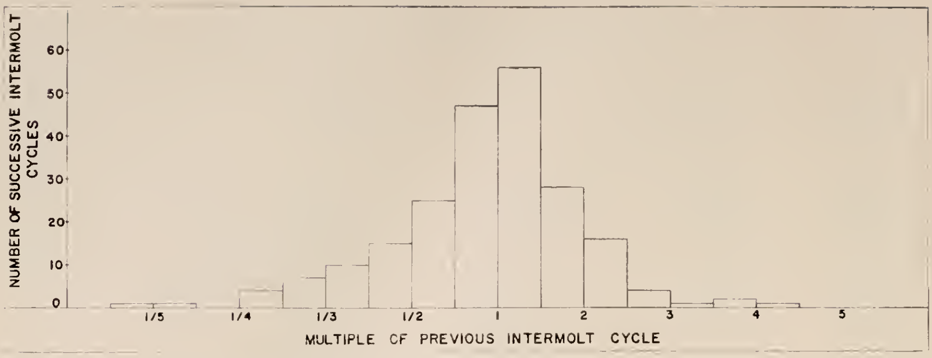


FIGURE 10. Frequency distribution of the relative durations of successive intermolt cycles of individual *Balanus amphitrite*. The duration of each intermolt cycle is calculated as a multiple of its previous cycle.

less than twelve hours to two days and Stage D lasted from one to five days. Stage C was found to be the most variable; it may outlast the other stages, or, in animals with short intermolt cycles, it may last less than one day. In terms of a percentage of an intermolt cycle, the stages also showed a large variation. Thus, Stage A made up less than 2 per cent; Stage B from 5 to 20 per cent; Stage C from 20 to 70 per cent; and Stage D from 20 to 40 per cent of the cycle.

Duration of the intermolt cycle

To analyze statistically the duration of the intermolt cycle of *B. amphitrite* 109 adult specimens were followed individually through three successive cycles (four ecdyses) from March to May, 1972. The durations of the intermolt cycles (Fig. 9) varied from 1.5 to 23.5 days. No significant difference was found between the frequency distributions of the three cycles (Kruskal-Wallis Test) showing that the population was stable throughout the experimental period. The three cycles when summed gave a mean duration of 8.3 days and a median of 7.6 days. Statistical tests for skewness and kurtosis showed that the distributions were significantly ($P < 0.001$) leptokurtic and positively skewed.

Each of the second and third intermolt cycles was calculated as a multiple of its previous cycle. These data (Fig. 10) show that the durations of successive intermolt cycles of individual animals also had a large variation. There was no correlation between the durations of successive intermolt cycles, the correlation coefficient being -0.08. Of the 218 successive cycles recorded, 107 had shorter and 108 had longer second cycles; 3 had identical intermolt cycle durations.

No correlation was found between the size (rostro-carinal diameters 5–10 mm) of the animal and the duration of the intermolt cycle. Also, ecdysis was not correlated with either the scotophase or photophase.

DISCUSSION

Two exoskeletal layers are visible in the cirri of *Balanus amphitrite* by light microscopy. Based on the following we assume the layers to be homologous with

TABLE III

Exocuticle thickness expressed as a percentage of the corresponding procuticle in the 13th segment of the inner rami of the right 4th, 5th, and 6th cirri of Balanus amphitrite *in various intermolt stages*

Intermolt stage	6th cirrus	5th cirrus	4th cirrus	Mean
B_1	68	68	70	69
B_1	67	72	74	71
B_1	69	68	65	67
B_2	57	51	59	56
C	36	35	35	35
C	35	39	38	37
C	44	42	44	43
C	31	30	35	32
C	34	33	32	33
D_1	33	31	33	32
D_1	49	46	47	47

the exocuticle and endocuticle of the arthropod procuticle. In the arthropods the exoskeleton, in general, is composed of three layers, a thin outermost epicuticle, the exocuticle, and the endocuticle (Hackman, 1971). By electron microscopy a three-layered exoskeleton is also found in *B. improvisus* (S. Koulish, Richmond College, Staten Island, New York, personal communication). The thickness of the outermost layer of this exoskeleton is below the resolving power of the light microscope so that by light microscopy only two layers would be visible. The sequence of formation and resorption of the exoskeletal layers in *B. amphitrite* during the intermolt cycle (Fig. 1) further supports the homology. The outer exoskeletal layer is discernible from early postecdysis and does not change in thickness during the intermolt cycle. This is consistent with the behavior of the arthropod exocuticle (Hackman, 1971). The main portion of the inner exoskeletal layer in *B. amphitrite* is secreted in postecdysis and resorbed in proecdysis. A small portion may also be secreted in proecdysis. This corresponds with the sequence of secretion and resorption of the arthropod endocuticle (Hackman, 1971).

The structural changes in the integument of *B. amphitrite* during an intermolt cycle are similar to those described for malacostracans (Passano, 1960; Yamaoka and Scheer, 1970). Drach's (1939, 1944) subdivision of the intermolt cycle is thus found to be applicable to cirripeds. In malacostracans post- and interecdysial stages are mainly characterized by the increasing rigidity of the exoskeleton, and the stages are identified by comparing the rigidity of various regions of the carapace (Drach and Tchernigovtzeff, 1967). Small size and the presence of the shell in cirripeds make palpation an impractical method for identifying the corresponding intermolt stages in these animals. Bocquet-Vedrine (1965) was unable to distinguish Stages B and C in *Elminius modestus*. We found, however, that in *B. amphitrite* these stages can be identified directly by microscopic examination of the exoskeleton of cirri mounted in glycerin jelly. The formation of the endocuticle is reflected in an increasing thickness of the procuticle and in a decreasing exocuticle percentage (Fig. 1). The criteria for the proecdysial stages in *B. amphitrite* are consistent with Drach's method (as noted by Bocquet, 1956, for stages D_1 and D_2 in

Chthamalus stellatus) except that resorption of the endocuticle is, again, observed directly rather than identified by palpation. The synchrony in the integumental changes allows the random use of the rami and of the segments within a ramus for intermolt staging. It is recommended, however, that the middle segments of the rami be used because of the conveniences of their size and the thickness of the exoskeleton.

It is difficult to obtain animals in Stage A because of its short duration. The best method of obtaining animals in this stage is to utilize animals within one hour of ecdysis. It should be noted, however, that the external presence of an exuvium attached to an animal is not, by itself, a suitable criterion for a recent ecdysis. We have observed that exuviae may remain attached to *B. amphitrite* in the laboratory for over three days after ecdysis. Likewise, we have observed *B. amphitrite* with exuviae attached in the field throughout the period of air exposure at low tide. Thus, the best method of obtaining newly molted animals is to make frequent observations of isolated individuals in Stage D_2 or D_3.

The frequency distribution of the durations of the intermolt cycles of a population of *B. amphitrite* has a large variability and is leptokurtic and positively skewed (Fig. 9). This type of distribution may be an intrinsic feature of the population since environmental factors were held constant and since successive intermolt cycles had similar distributions. It would appear that a minimum amount of time is needed for an animal to complete its exoskeleton following ecdysis and to prepare for its forthcoming ecdysis, with the majority of the animals in a population molting soon after the minimum time limitation. It is also interesting to note that despite the large variation in the durations of successive intermolt cycles of individual animals (Fig. 10), the variation in the population as a whole is such that the original distribution is maintained through successive cycles (Fig. 9). This phenomenon is also indicated by the nearly normal form of the frequency distribution of Figure 10. The large variability in the successive cycles of individual *B. amphitrite* does not support the notion of fast and slow molters as was proposed for *B. balanoides* (Patel and Crisp, 1961).

The large variability in the duration of the intermolt cycle and its stages in adult *B. amphitrite* stresses the inadequacies of the timed interval method as a procedure for intermolt staging in cirripeds. If the timed interval method was to be applied the animals could be in several different intermolt stages at any given interval after ecdysis. This could introduce large variation in the data and thus obscure any intermolt stage dependency. Moreover, the present method eliminates the requisite of a knowledge of the prehistory of the individual animal that is necessary for the timed interval method. This allows the use of animals chosen at random from either laboratory or field populations and thus broadly widens the scope of intermolt cycle related studies in cirripeds. The present method should be applicable to all thoracic cirripeds as long as the cirri are transparent enough to allow microscopic examination and as long as the proper exocuticle percentages are calculated for each species.

We wish to thank Dr. John D. Costlow for his continuous interest and encouragement during this study. We also thank Dr. Sasha Koulish for allowing us to examine his electron micrographs. H. J. Fyhn acknowledges support from the Norwegian Research Council for Science and the Humanities and from the American-Scandinavian Foundation. This study was supported by a contract, NR-104–194, between Duke University and the Office of Naval Research.

SUMMARY

The intermolt cycle of the barnacle *Balanus amphitrite* is divided into three postecdysial, one interecdysial, and four proecdysial stages based on integumental changes in the cirri. Stage A is characterized by a seemingly single-layered exoskeleton and tortuous cirri. Stages B_1, B_2, and C are characterized by the increasing thickness of the endocuticle. Stage D_0, D_1, D_2, and D_3 are characterized by the progression of setogenesis, formation of the new exoskeleton, and resorption of the old endocuticle. The durations of the intermolt stages have a wide variability. The integumental changes both within and between the rami of an animal progress synchronously. The criteria allows the use of live, intact animals taken at random from laboratory or field populations. The method is assumed to be applicable to all thoracic cirripeds as long as the exoskeleton is transparent enough to allow microscopic examination.

The duration of the intermolt cycles of adult specimens of *B. amphitrite* varied from 1.5 to 23.5 days under constant conditions (23° C, 30‰, LD 12:12). The distributions of the cycles are significantly ($P < 0.001$) leptokurtic and positively skewed. There is no correlation between the durations of successive intermolt cycles of individual animals.

LITERATURE CITED

Barnes, H., and M. Barnes, 1963. The relation of water uptake and oxygen consumption of body tissues to the moulting cycle in *Balanus balanoides* (L.). *Crustaceana,* **6**: 62–68.

Bocquet, J., 1956. Corrélation entre le rythme des marées et la mue d'une population de Chthamales. *C. R. Acad. Sci. Paris,* **243**: 2159–2162.

Bocquet-Vedrine, J., 1965. Etude du tégument et de la mue chez le Cirripède Operculé *Elminius modestus* Darwin. *Arch. Zool. Exp. Gén.,* **105**: 30–76.

Costlow, J. D., 1963. Molting and cyclic activity in chromatophorotropins of the central nervous system of the barnacle, *Balanus eburneus. Biol. Bull.,* **124**: 254–261.

Costlow, J. D., and C. G. Bookhout, 1953. Moulting and growth in *Balanus improvisus. Biol. Bull.,* **105**: 420–433.

Costlow, J. D., and C. G. Bookhout, 1956. Molting and shell growth in *Balanus amphitrite niveus. Biol. Bull.,* **110**: 107–116.

Drach, P., 1939. Mue et cycle d'intermue chez les Crustacés Decapodes. *Ann. Inst. Océanogr.,* **19**: 103–391.

Drach, P., 1944. Etude préliminaire sur le cycle d'intermue et son conditionnement hormonal chez *Leander serratus* (Pennant). *Bull. Biol. Fr. Belg.,* **78**: 40–62.

Drach, P., and C. Tchernigovtzeff, 1967. Sur la méthode de détermination des stades d'intermue et son application générale aux Crustacés. *Vie Milieu,* **18**: 595–609.

Hackman, R. H., 1971. The integument of Arthropoda. Pages 1–62 in M. Florkin and B. T. Scheer, Eds., *Chemical Zoology,* Vol. VI, *Arthropoda,* Part B. Academic Press, New York and London.

Harding, J. P., 1962. Darwin's type specimens of varieties of *Balanus amphitrite. Bull. Brit. Mus. (Natur. Hist.) Zool.,* **9**: 273–296.

Hubschman, J. H., 1962. A simplified azan process well suited for crustacean tissue. *Stain Technol.,* **37**: 379–380.

Passano, L. M., 1960. Molting and its control. Pages 473–536 in T. H. Waterman, Ed., *The Physiology of Crustacea,* Vol. I. Academic Press, New York and London.

Patel, B., and D. J. Crisp, 1961. Relation between the breeding and moulting cycles in cirripedes. *Crustaceana,* **2**: 89–107.

Shimony, T., and R. F. Nigrelli, 1972. Studies on arylsulphatases in the barnacle *Balanus eburneus. Mar. Biol. (Berlin),* **14**: 349–358.

Sokal, R. R., and R. F. Rohlf, 1969. *Biometry.* W. H. Freeman and Co., San Francisco, 776 pp.

Yamaoka, L. H., and B. T. Scheer, 1970. Chemistry of growth and development in crustaceans. Pages 321–341 in M. Florkin and B. T. Scheer, Eds., *Chemical Zoology,* Vol. V, *Arthropoda,* Part A. Academic Press, New York and London.

Reference: *Biol. Bull.*, **145**: 323–339. (October, 1973)

THE RESPIRATORY ADAPTATIONS OF THE PODIA AND AMPULLAE OF ECHINOIDS (ECHINODERMATA)[1]

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Early work on the structure of echinoid podia and ampullae consisted of verbal descriptions and line drawings (Bather, 1900; Chadwick, 1900; Ludwig, 1904; MacBride, 1909; Cuenot, 1948; Hyman, 1955), some of which were highly schematic. Nichols (1959a, 1959b, 1961) has made photomicrographs showing the wide variety of adaptations of the terminal suckers of several echinoids. Most recently, Kawaguti (1964, 1965), and Coleman (1969) have investigated the structure of the wall of the tube feet and ampullae of echinoids using electron microscopy. Because the podia are the primary respiratory structure of echinoids (Farmanfarmaian, 1959, 1966, 1968; Steen, 1965), a systematic search for respiratory adaptations in the structure of echinoid podia was undertaken (Fenner, 1971).

The podium-ampulla system of most echinoids differs from that of asteroids and holothuroids in two respects. First, the connection between the echinoid podium and ampulla consists of two pores through the body wall instead of one. Recently, Yoshida (1966), and Coleman (1969) showed a septum dividing the base of the tube foot of *Diadema,* each half of the lumen being served by one of the pair of pores through the body wall. An exception to the rule of two pores per podium is the arrangement found in the accessory tube feet on the oral surface of the sand dollar *Echinocyamus,* which have only one pore passing through the body wall (Nichols, 1959b). Secondly, the echinoid ampulla has been reported to be crossed by strands of tissue (Ludwig, 1904; Cuenot, 1948; Hyman, 1955), or septae (Kawaguti, 1965), unlike the asteroid or holothuroid ampulla.

The non-locomotor podia on the aboral surface of several echinoids (*Cidaris* and *Echinus:* Nichols, 1961; clypeastroids: Hyman, 1955; and spatangoids: Hyman, 1955, and Chesher, 1969) are believed to serve a respiratory function. Nichols (1959b) presented the histology of the respiratory podia (petaloids) of the clypeastroid, *Echinocyamus.* Ciliary currents within the podia contribute to respiratory gas transport (Faramanfarmaian, 1966). The spatangoids (Chesher, 1969) have a counter-flow between the ciliary currents within the podia and the ciliary currents outside the podia; such a counter-current should enhance exchange of gases across the surface of the respiratory podia.

This study was undertaken to systematically search for respiratory adaptations in echinoid podia and ampullae, and to clarify our knowledge of the morphology of echinoid podia and ampullae.

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MATERIALS AND METHODS

Specimens were obtained from Gulf Specimen Co., Dr. J. Morin, Dr. J. McCauley, B. Madden, Dr. R. Wiggley, Dr. H. Sanders, Dr. R. Fay, Dr. P. Kier, Dr. W. Durham, the Marine Biological Laboratory Supply Department, and the Smithsonian Institution. The taxonomy of the species studied is presented in Table I.

Ciliary currents were mapped by watching the movement of carbon particles outside of the podia and ampullae, and free-floating pigmented coelomyctes within podia and ampullae. For histology, living specimens were relaxed in 7% MgCl before podia were removed and fixed in 10% formaldehyde or Bouin's Fixative (Gurr, 1962). Decalcification was accomplished by the actic acid in the Bouin's, or 10% HCl after formaldehyde fixation. Tissues were dehydrated through a series of alcohols and embedded in parafin. Sections were cut at 8–15 microns and stained with Haematoxylin-Eosin (Gurr, 1962), Mallory's Haematoxylin (Grey, 1966), or Hubschman's (1962) modification of the Mallory triple connective-tissue stain.

TABLE I

The classification of the species studied, from Mortensen (1928–1951). Asterisk indicates living specimen studied

- Class Echinoidea
  - Sub-class Regularia
    - Order Echinothuroida
      - Family Echinothuridae
        - *Sperosoma giganteum*
        - *Phormosoma plascentei*
    - Order Cidaroida
      - Family Cidaridae
        - *Cidaris abysicola*
        - *Eucidaris tribuloides**
    - Order Stirodonta
      - Family Arbaciidae
        - *Arbacia punctulata**
        - *Tetrapygus niger*
      - Family Saleniidae
        - *Salenia goesiana*
      - Family Stomopheustidae
        - *Stomopneustes variolaris*
      - Family Phymosomatidae
        - *Glyptocidaris crenularis*
    - Order Camarodonta
      - Family Toxopneustidae
        - *Lytechinus variagatus**
        - *Tripneustes gratilla*
      - Family Temnopleuridae
        - *Salmacia alexandri*
      - Family Strongylocentrotidae
        - *Strongylocentrotus purpuratus**
        - *Strongylocentrotus droebachiensis**
        - *Allocentrotus fragilis*
    - Order Camarodonta (continued)
      - Family Echinidae
        - *Echnius affinis*
      - Family Echionometridae
        - *Echinometra mathei*
        - *Heterocentrotus mammilatus*
      - Family Parasaleniidae
        - *Parasalenia gratiosa*
    - Order Aulodonta
      - Family Diadematidae
        - *Centrostephenus coronatum*
      - Family Micropygidae
        - *Microgyga tuberculata*
      - Family Aspidodiadematidae
        - *Plesiodiadema indicum*
      - Family Pedinidae
        - *Caenopedina indica*
  - Sub-class Irregularia
    - Order Clypeastroida
      - Family Scutellidae
        - *Mellita quinquesperforata**
        - *Echinarachnius parma**
        - *Dendraster excentricus**
    - Order Spatangoida
      - Family Schizasteridae
        - *Brisaster latifrons*
      - Family Loveniidae
        - *Lovenia cordiformis*
      - Family Brissidae
        - *Brissopsis lyrifera*
      - Family Hemiasteridae
        - *Hemiaster expergitus*

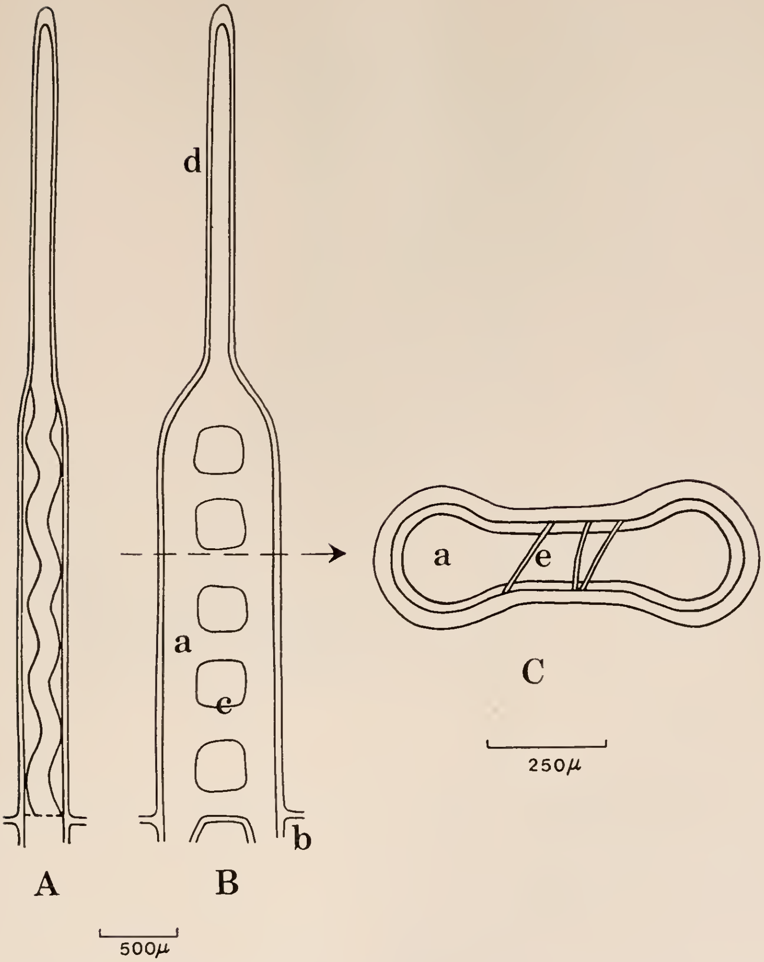

FIGURE 1. Respiratory podium of *Eucidaris tribuloides;* (A.) edge view; (B.) side view; (C.) cross section; (a.) marginal tube; (b.) test; (c.) thin, rippled area; (d.) distal tube; (e.) strands of connective tissue. The actual sizes of podia and ampullae vary considerably with the size of the animal, location of podia on animal, and state of contraction of podium.

RESULTS

Order Echinothuroida

Two species in this order were examined: *Sperosoma giganteum* and *Phormosoma plascentei.* There are two pores in the body wall for each podium, and both podia and ampullae are hollow. Podia on the oral surface, which terminate in suckers, fit the classical description of tube feet (Bather, 1900; Chadwick, 1900; Ludwig, 1904; MacBride, 1909, Cuenot, 1948; Hyman, 1955). Podia on the aboral surface, while shaped like tube feet, terminate bluntly without a sucker.

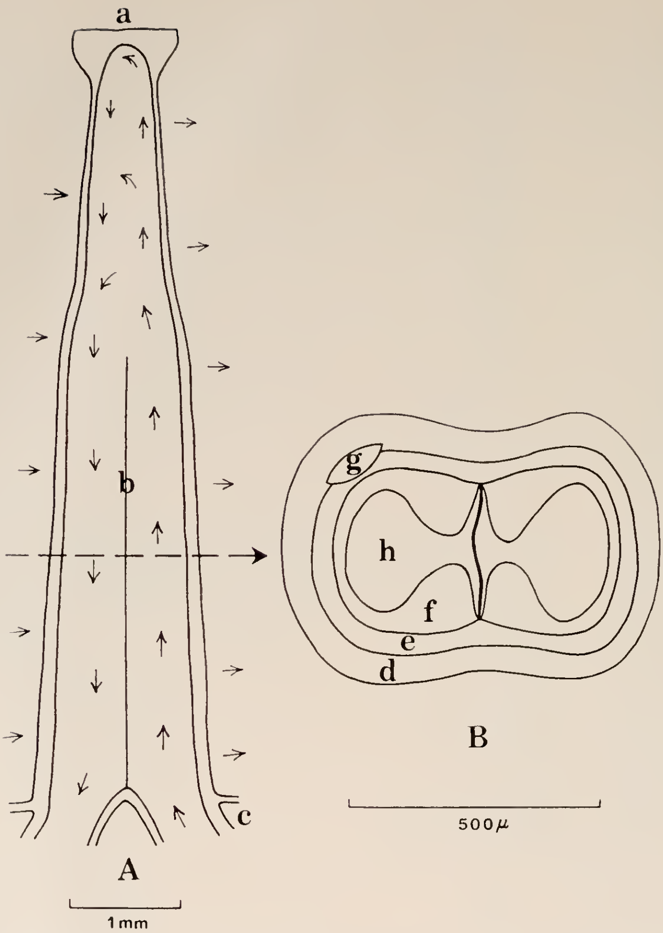

FIGURE 2. Tube foot of *Strongylocentrotus purpuratus;* (A.) whole tube foot seen from side; (B.) cross section from "double-barreled" region; (a.) sucker; (b.) septum; (c.) test; (d.) epithelium; (e.) connective tissue layer; (f.) muscle; (g.) nerve; (h.) lumen. Arrows indicate ciliary currents.

Order Cidaroida

Cidaris abyssicola and *Eucidaris tribuloides* have two types of podia, both served by pairs of pores in the test. Podia on the oral surface are hollow tube feet with terminal suckers. Podia on the sides and aboral surface are flattened, respriatory podia lacking suckers (Fig. 1). There is a channel along each edge and a thin, rippled area in between. The distal half is a thin, bluntly ending tube. The lumen is crossed by a few large strands of connective tissue. Often the respiratory podia are partially or wholly retracted beneath the ambulacral spines. When preserved without prior relaxation, these podia retract until they form a blunt triangle. The ampullae of *C. abyssicola* are flattened sacs, the lumen being crossed by septae, similar to the camarodont ampullae described below.

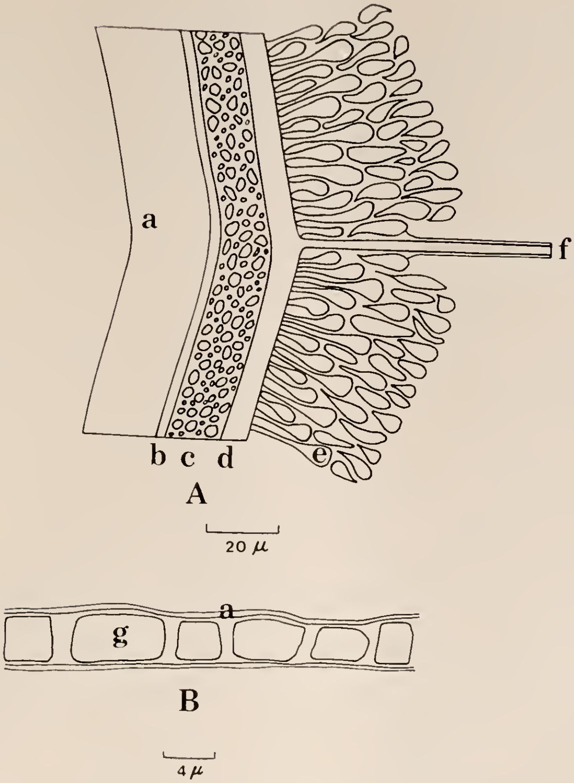

Figure 3. Details of the tube foot wall and septum of *S. purpuratus*. (A.) Detail of the junction of the connective tissue in the podial wall and the septum, from a cross section of the podium; (B.) detail of the septum sectioned parallel to the length of the tube foot; (a.) epithelium; (b.) outer, circular layer of connective tissue; (c.) middle, longitudinal layer of connective tissue; (d.) inner, circular layer of connective tissue; (e.) muscle cells; (f.) septum; (g.) cross section of strand of connective tissue.

Order Camarodonta

Families Strongylocentrotidae, Toxopneustidae, Temnopleuridae, and Echinidae. The seven members of these families that have been studied (*Strongylocentrotus purpuratus, S. drobachiensis, Allocentrotus fragilis, Lytechinus variagatus, Tripneustes gratilla, Salmacia alexandri,* and *Echinus affinis*) have virtually identical morphology and will be treated together.

In all seven species, there are two pores through the body wall for each tube foot. A septum, continuous with the wall separating the two pores of the test, runs halfway up the center of the podium (Fig. 2). In a cross section of the podium (Figs. 2B, 3A), it can be seen that the septum is continuous with a layer of tissue in the wall of the tube foot. The septum is continuous with the inner of the three adjacent layers of connective tissue in the wall of the podium (Fig. 3A). Each layer is made up of strands 2–6 microns in diameter. The inner and outer layers run circularly while those in the middle layer are longitudinal strands.

Strands in the septum form a single layer running across the lumen. A lightly staining layer less than one micron thick covers the septal strands and bridges the gaps between them (Figure 3B). The strands probably correspond to the bundles of collagen fibers, and the thin covering to the epithelium lining the podial lumen, which Kawaguti (1964) found in electron micrographs. Coleman (1969) found that the septum in the podia of *Diadema antillarum* consisted of collagen, a single cell layer thick. The buccal podia do not appear to have septae. Podia on the aboral surface have septae as described. The 8 podia nearest the aboral pole in each ambulacral row are so short that they do not project above the test. These have no septae or suckers. The most aboral 8 podia were examined closely only in *S. droebachiensis.*

The ciliary current within the tube foot moves toward the proximal end of the podium on the side nearest the radial canal, and toward the distal end on the other side (Cuenot, 1948). In the proximal half of the tube foot these two currents are separated by the septum, but in the distal half there is no dividing septum and mixing by eddies may occur. The ciliary current outside the tube foot moves across it, and thus does not run counter to the current inside.

The ampullae are flattened, leaf-like sacs that connect by way of the pore pairs to the podia and by way of a small tube (Fig. 5Ab) guarded by a one way valve (Nichols, 1966) to the radial canal. The ampullae lie like pages of a book, one row on each side of the radial canal in each ambulacrum. The distance separating adjacent ampullae is about equal to the thickness of one ampulla (about 0.2 mm). The pores pass through the test at angles up to 45°, such that the pores are closely adjacent to each other where they connect with the podium, but more widely separated where they connect with the ampulla. The lines seen within the ampulla correspond to septae connecting the walls (Figs. 4B, 4C). The septae consist largely of connective tissue, but the presence of muscular tissue could not be denied or confirmed. Only *A. fragilis* has ampullae that are connected to each other by strands attached to the outer edge of the ampullae.

The septae guide the ciliary currents within ampullae (Fig. 4B). The ciliary current outside the ampullae runs counter to that within the ampullae. This is true of flattened ampullae divided by septae in all species examined alive.

Families Echinometridae, Parasaleniidae. Echinometra mathei, Heterocentrotus mammilatus, and *Parasalenia gratiosa* have tube feet on their oral surfaces which are similar to those of members of the other camarodont families: they are tall, and each has a sucker and a septum. Strands in the septum of the podia of *P. gratiosa* are not connected by an epithelium, but elsewhere the lumen is lined with a thick epithelium covering the muscular layer. The aboral surface (and equatorial areas of *H. mammilatus*) has greatly shortened, slightly flattened podia with rounded distal ends lacking suckers. The presence or absence of septae or strands within these podia was not determined. All of the ampullae are similar to the other camarodonts.

Order Aulodonta

The four aulodont species studied (*Centrostephanus coronatum, Micropyga tuberculata, Plesiodiadema indicum,* and *Caenopedina indica*) have nearly identical podial morphology. Podia on the oral surface are suckered tube feet with septae, and closely resemble the tube feet of the oral surface of camarodonts. Podia on

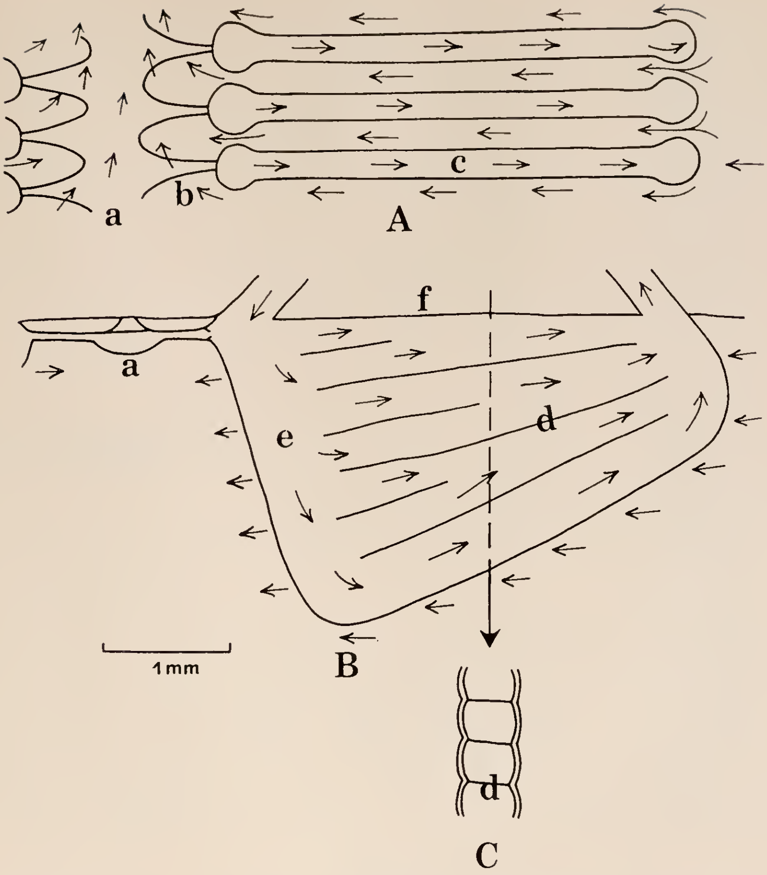

Figure 4. Ampullae of *S. purpuratus*; (A.) a row of ampullae lined up in the ambulacral areas as seen from the coelomic side of test; (B.) a single ampulla viewed from the side; (C.) cross section of the ampulla; (a.) radial canal; (b.) branch connecting radial canal and ampulla; (c.) ampulla; (d.) septae; (e.) marginal tube; (f.) test. Arrows indicate ciliary currents.

the aboral surface of all four aulodont species are bluntly-ending (no suckers), thin-walled podia with septae. The aboral podia are roughly as tall as the oral podia (and thus much taller than the aboral podia of the echinometrids and parasalenid studied).

The ampullae of two species, *C. coronatum* and *M. tuberculata*, were studied. Ampullae of both species consist of flattened sacs, the lumen of which is crossed by septae or "bands." In *M. tuberculatum*, "bands" crossing the lumen are arranged in definite rows.

Order Stirodonta

The only stirodont available alive was *Arbacia punctulata*, which will be discussed first. Specimens of *A. punctulata* have four types of podia. Nearest the

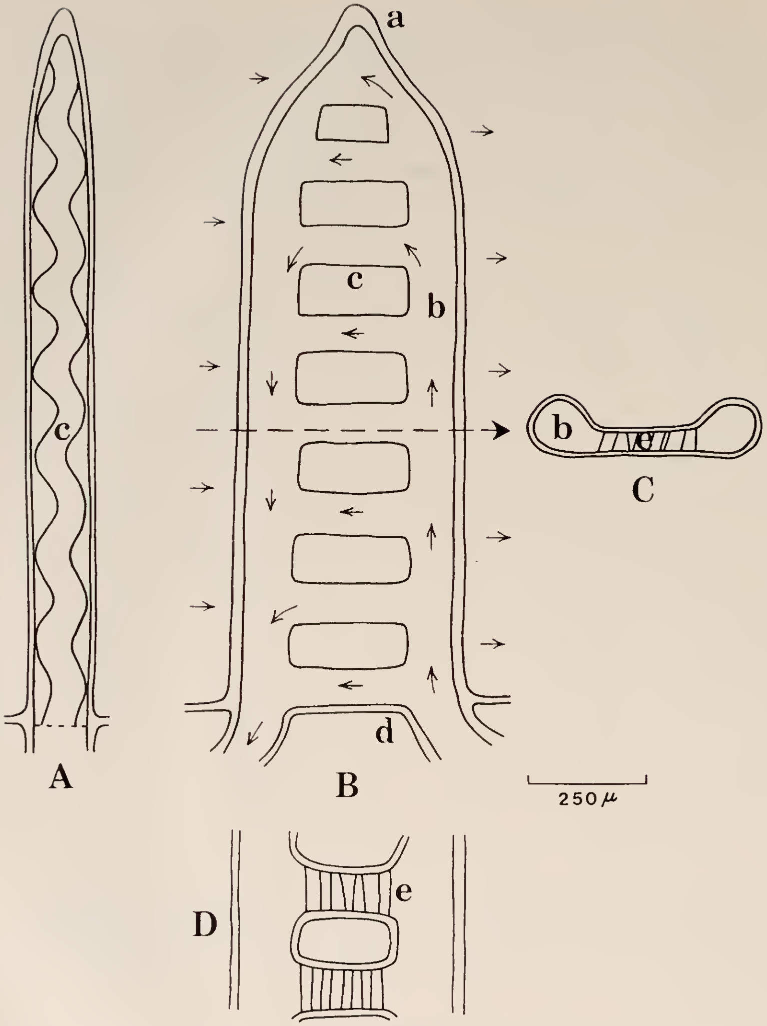

Figure 5. Respiratory podium of *Arbacia punctulata;* (A.) podium viewed from the edge; (B.) podium viewed from the side; (C.) cross section of the podium; (D.) longitudinal section of the podium; (a.) apex; (b.) marginal tube; (c.) thin, rippled area; (d.) test; (e.) strands of connective tissue. Arrows indicate ciliary currents.

aboral pole are flattened respiratory podia that lack suckers. At the animal's equator are long tube feet with small suckers, which may be sensory. These tube feet continually wave about, and do not attach to the substrate during locomotion. When they touch an individual of the same species, the animal locomotes away from the other individual. The buccal podia resemble the camarodont's buccal podia. The locomotor tube feet are located between the long podia and the buccal podia. The locomotor tube feet are moderately long (more so than the oral spines), have a large sucker, and no strands or septum.

The respiratory podia have two tubes, one running along each edge. The tubes are connected at the apex of the podium and by a thin, rippled area between the tubes (Fig. 5). Thus, a cross section in the middle of the petaloid is dumbbell-shaped (Fig. 5C). The two walls of the rippled area are held a fixed distance apart by thin strands of connective tissue crossing the lumen. Londitudinal sections show that the connective tissue is in the form of strands and not septae (Fig. 5D). Ciliary currents within the podium move toward the ampulla in the tube nearest to the radial canal and away from the ampulla on the other side. The current crosses the thin, rippled area along the arrows shown in Figure 5B. On the outside of the podium, the ciliary current passes across the podium counter to the current inside the thin, rippled area of the podium (Fig. 5B). The most aboral of these respiratory podia have a hollow tubular region on the distal end like those of the cidarids (Fig. 1).

The long, sensory podia have a small sucker, a hollow tubular section and a double-barreled section near the base. Instead of a septum, strands of connective tissue similar to those in the petaloids separate the two barrels near the base of the tube foot. In the sensory and locomotor podia, the ciliary currents within the tube foot move proximally on the side nearest the radial canal, and distally on the opposite. Some mixing between the two currents occurs. Each podium is served by two pores in the test.

The ampullae are also differentiated. All of the ampullae except those nearest the mouth closely resemble the camarodont ampullae (Fig. 4). The ten or so ampullae in a row nearest the mouth do not have septae, and are not flattened but irregular in shape. Unlike the other ampullae, those closest the mouth do not have their ciliary currents organized in a counter-current fashion.

Tetrapygus niger (also in family Arbaciidae) has hollow tube feet with suckers on its oral surface like *A. punctulata*. Some of the podia on the aboral surface closely resemble those on the aboral surface of *A. punctulata*. Others are more flattened and blunt on the distal end, but are crossed by strands like the rippled podia.

Salenia goesiana (family Saleniidae) has spines which closely resemble those of the cidarids: large primary spines surrounded by smaller spines, and two rows of spines between the two rows of podia in each ambulacrum. These latter spines are somewhat flattened and project over the podia. The preserved aboral podia are small and blunt, resembling the preserved podia of cidarids. The tube feet on the oral surface are hollow like those of the cidarids.

Stomopneustes variolarus and *Glyptocidaris crenularis*, in contrast to the previous stirodonts considered, have aboral tube feet with suckers, and all tube feet have septae like the camarodont podia.

Order Clypeastroida

Three species, all in family Scutellidae, were examined: *Mellita quinquesperforata, Dendraster excentricus,* and *Echinarachnius parma*. The podia of all three species are differentiated into two distinct types. Located on the aboral surface are large respiratory podia (petaloids) arranged in very definite ambulacral rows which in the cleaned test form five petals. The small (accessory) tube feet, however, are scattered throughout the ambulacral areas on the oral surface (most dense in the

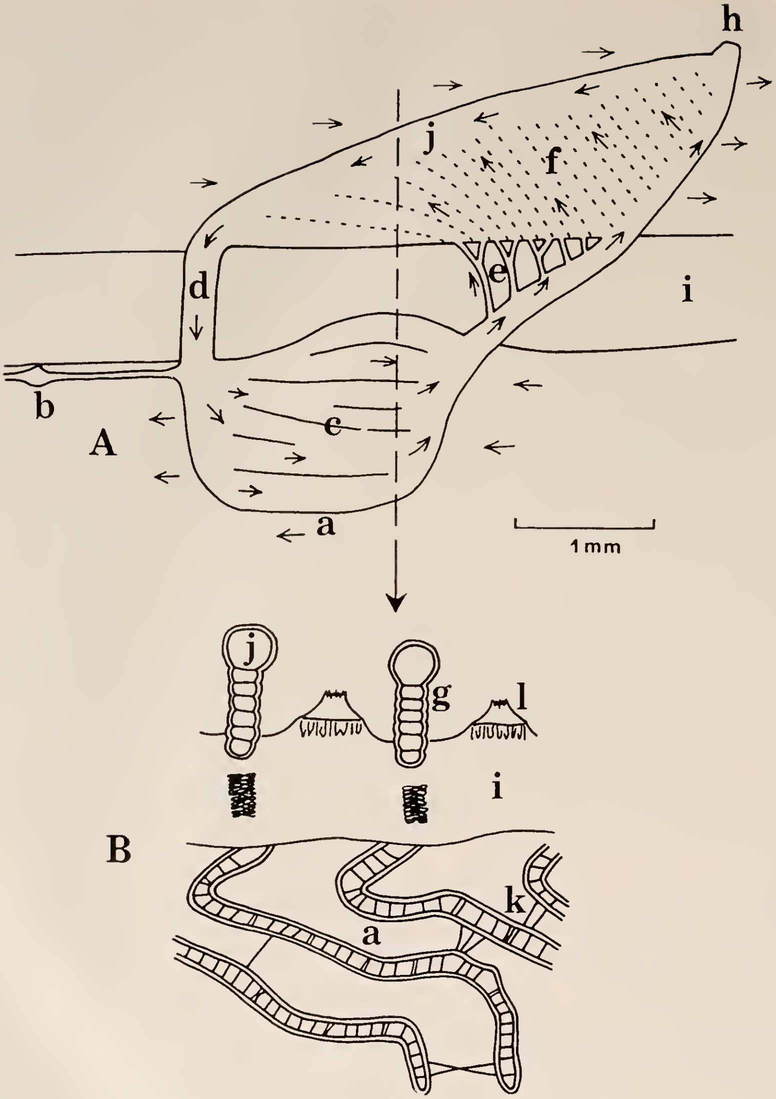

Figure 6. The respiratory podium and ampulla of *Dendraster excentricus;* (A.) viewed from the side; (B.) seen in cross section; (a.) ampulla; (b.) radial canal; (c.) septum; (d.) pore through the test; (e.) secondary pores through the test; (f.) rows of strands; (g.) petaloid (respiratory podium); (h.) knob; (i) test; (j.) marginal tube; (k.) strands connecting ampullae; (l.) bases of spines.

ambulacral grooves) and edges of the sand dollar. In *E. parma* and *D. excentricus* they exist on the aboral surface between the two rows of petaloids in each petal (ambulacral row). In that area only, they are arranged in rows in line with the petaloids. In *D. excentricus,* the ambulacral grooves of the oral surface continue around on the aboral surface as ridges: some running within the petals, some between.

The accessory tube feet of all three species are small, thin tubes terminating in adhesive bulbs such as in *Echinocyamus pusillus* (Nichols, 1959b). The largest accessory tube feet on *D. excentricus* have a sucker instead of a bulb on the end. The shaft has a hollow lumen served by a single pore in the test. No ciliary currents exist within or around the tube feet. However, coelomocytes (and thus the fluid in the lumen) move distally during extension of the tube feet, and proximally during their retraction.

The petaloids are long parallel to the test and protrude from the test as far as the spines between which they are found. The end farthest from the radial canal protrudes farthest from the test, and has a small lump resembling the adhesive bulb at the end of the accessory tube feet. A large tube runs along the top edge of the petaloid, and connects to the pore(s) farthest away from the radial canal by small "tubes" (Fig. 6). The lumen of the petaloid below the large tube is completely divided up into the small "tubes" by rows of strands of connective tissue. Nichols (1959b) reports that the petaloids of *Echinocyamus pusillus* are crossed by 4–8 strands per podium. Free-floating coelomocytes were never observed passing from one "tube" to another, but always moving within a single "tube." Thus, the rows of strands are quite effective in directing ciliary currents. The ciliary currents on the outside of the petaloid move away from the radial canal, counter to the current within the petaloid.

The passage of the tubes through the test was studied by breaking and examining cleaned, dried tests. The pore passing through the test nearest the radial canal is unitary, except in *D. excentricus*, where it may split into two as it approaches the inner surface of the test. In *D. excentricus* and *M. quinquesperforata* (*E. parma* was not closely examined), the pore farthest from the radial canal gives off one to eleven tubes on the side nearer the radial canal as it approaches the outer surface of the test (Fig. 6Ae). The same arrangement has been found in fossil scutellids (Schaffer, 1962). The ampullae serving the petaloids are flattened sacs divided by septae. In all three species, the ampullae are bent so as to lie at an angle, and are connected to each other by strands (Fig. 6B). Ciliary currents in the petaloids' ampullae move as in camarodont ampullae. The ampullae connected to the accessory tube feet are simple, round or elongated sacs, without septae. On the aboral surface of *E. parma* and *D. excentricus* they are located along the branch canals connecting the radial canal and petaloid ampullae. On the oral surface of these two species, these small ampullae are located on branches leading from the radial canal, many ampullae on each branch. In *M. quinquesperforata*, however, there are ampullae visible only on the branches nearest the mouth. Farther from the mouth the branches enter the test without ampullae attached to them. The branches pass horizontally for about 2–10 mm in the system of "microcanals" (Schaffer, 1962). Durham (1966) reports that Wagner found ampullae for the accessory tube feet within the microcanals of *Encope*. No ciliary currents were seen within or around any of the simple ampullae in the present study.

Order Spatangoida

The four species examined (*Brisaster latifrons, Brissopsis lyrifera, Hemiaster expergitus,* and *Lovenia cordiformis*) have nearly identical podial morphology and

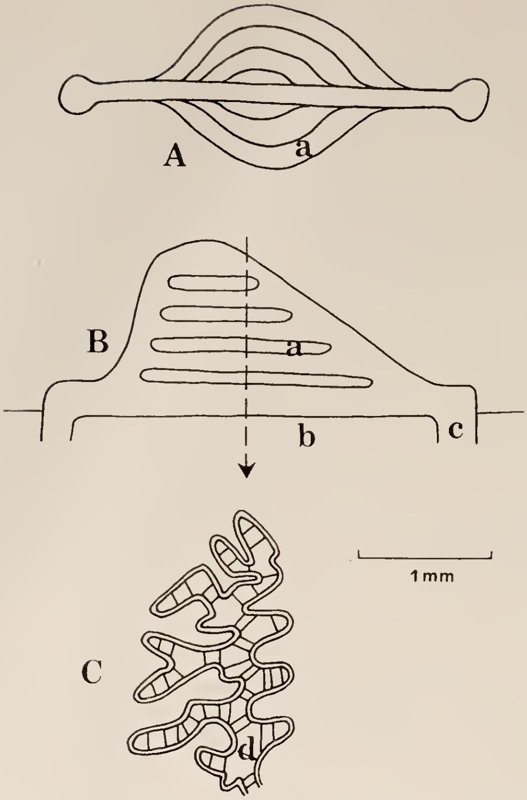

FIGURE 7. The resipratory podium of *Brisaster latifrons;* (A.) as seen from above; (B.) as viewed from the side; (C.) cross section; (a.) "lamellae" or "ripples"; (b.) test; (c.) pore through test; (d.) strands or septae.

will be treated together. Heat urchins (spatangoids) have highly differentiated podia. The tube feet of the anterior petal are used in building the respiratory tunnel to the surface of the substrate. Respiratory petaloids are found in the other four petals. Tube feet surrounding the mouth and anus are used in ingestion and building the drainage canals, respectively.

The tunnel-building tube feet of the anterior petal have a hollow lumen served by a pair of pores in the test. The ampullae connected to those tube feet are simple, hollow sacs. The oral and anal podia and their ampullae are similar to the tunnel-building tube feet, except they are each served by a single pore in the test.

The respiratory petaloids are triangular-shaped, stretched between their two pores. The sides of the larger petaloids are folded into "ripples" or "lamellae" (Fig. 7). The lamellae of *B. latifrons* and *B. lyrifera* alternate between the two sides (Fig. 7C), just as do the "ripples" of the cidarid and arbacid respiratory podia. The lamellae of *L. cordiformis* and *H. expergitus* are found on only one side of the petaloid, as shown for *Echinocardium cordatum* by Cuenot (1948).

Septae (or possibly rows of strands) consisting of connective tissue covered with epithelium divide the lumen and fix the separation of the walls. They probably channel the ciliary currents as in the clypeastroid petaloids. The petaloids are served by camarodont-type ampullae. The central area of some of these ampullae bend and fold out of the plane of the ampulla.

Discussion

In the variety of respiratory podia presented above, four principal adaptations are apparent which are absent in podia not specialized for respiration (*i.e.,* other podia in echinoids, podia of asteroids and holothuroids). First, there is the separation of the two ciliary currents within the podium; secondly the increase in surface area of the podia and ampullae; thirdly, the counter-current across the surfaces of podia and ampullae; and fourthly, the favorable positioning on the animal of podia specialized for respiration.

The separation of the ciliary current going from ampulla to podium from the return current is accomplished by the division of the pore passing through the test into two pores, each containing a single current. The great separation of the current entering the ampulla from that leaving is accomplished by the ampulla's flattened shape, and the current-guiding septae within. The currents entering and leaving the podium may be separated by a septum or by the flattened shape of the podium. In tube feet with septae, a counter-current across the septum may occur, reducing efficiency.

The surface area of some ampullae and podia is increased by their flattening. The podia of cidarids, arbacids, and spatangoids further increase their respiratory surface area by having their walls folded into ripples and lamellae. The walls of the flattened podia and ampullae are held together by the strands and septae of connective tissue which thus perform a function similar to the pillar cells in teleost (Hughes and Grimstone, 1965), and crustacean (Copeland, 1968) gill lamellae.

The ciliary currents inside and outside of flattened ampullae are arranged in a counter-current, as are the currents of flattened podia. The ciliary currents of tube feet with septae are not so arranged. Currents on the inside and outside of the podia move at right angles to each other. Ciliary currents provide the only ventilation of the highly specialized respiratory podia of the aboral surfaces of many echinoids. These podia are not used for locomotion, and (except for the cidarids) retract only if stimulated. Movement of the tube feet used for locomotion may contribute to ventilation.

The ciliary currents crossing the two rows of ampullae in an ambulacrum move from the inter-ambulacral area toward the radial canal, and then into the coelomic cavity. This is a more efficient arrangement than if the current passed over one row of ampullae, and then over the second before circulating into the cavity. After passing over one row of ampullae, the fluid has an increased oxygen content. If it then passed over the second row, the oxygen gradient across the (second row) ampullar walls would be less than if the fluid had not passed over the first row of ampullae before passing over the second row. The ciliary currents over respiratory podia are likewise advantageously arranged, and Paul (1968) has postulated a similar system for some fossil echinoderms.

The podia having the above three adaptations are invariably positioned favorably on the animal. Echinoids, with one known exception, keep their mouth toward the substrate, or pointed down if they burrow in the substrate. The flattened podia of cidarids, salenids, and arbacids are found on the aboral surface of these epifaunal species. The flattened podia of the clypeastroids and spatangoids are confined to the aboral surface of these infaunal species. By projecting away from the aboral surface, the respiratory podia miss the layer of oxygen-depleted water around the animal and its substratum. The single exception to the rule that all echinoids keep their mouth down or toward the substrate is the sand dollar, *Dendraster excentricus.* This sand dollar frequently "stands" on its anterior edge with that edge submerged in sand. It is eccentric such that the mouth and petals containing the respiratory podia are nearer the posterior (anus) than anterior edge. Thus, the resipratory podia are kept above the oxygen-poor sand.

The peristomeal gills of regular echinoids may be relatively undeveloped because they project down toward the substrate. The infaunal, irregular echinoids have no such peristomeal gills and depend entirely upon podia for respiratory structures. The podia of classes of echinoderms other than Echinoidea may not be anatomically specialized for respiration because alternative respiratory structures project upward into the water column.

It is not clear why *Stomopneustes variolaris, Glyptocidaris crenularis* and most camarodonts show no oral-aboral differentiation of podia. It seems likely that the suckered aboral podia are adequately efficient at respiration due to the septum, and in addition can function in locomotion. These suckered aboral podia may serve a food-catching function, may be used to cover the animal with objects for shade or camouflage, and/or serve to anchor the animal following dislodging before breakage by wave action can occur.

The type of ampulla is correlated with the resipratory specialization of the podium it serves. Thus, flattened podia and podia with septae have flattened ampullae with septae, but hollow tube feet have hollow ampullae. The single exception found is a few hollow tube feet on the oral surface of *Arbacia punctulata* which may have flat, septate ampullae. That the septae of the ampullae serve primarily to increase strength in protraction of locomotor tube feet is rejected by consideration of the spatangoids. Spatangoids (heart urchins) have flat, septate ampullae serving non-locomotor respiratory podia, and hollow ampullae serving tunnel-building, *etc.* tube feet, which require greater protraction force than respiratory podia.

None of the above discussion of increased respiratory efficiency of some podia due to several adaptations has been corroborated by actual measurements and comparisons of efficiency of the various podia. Many podia and other external surfaces have been shown to act as gas exchange surfaces even though they are not specialized for that purpose (Farmanfarmaian, 1966). The measurement of oxygen tensions at selected positions in and around podia and other respiratory structures might indicate the relative efficiency of those structures with and without separation of currents, presence of counter-currents, and other adaptations.

The septae and strands of podia and ampullae of all species examined have a common basic structure. All are composed of strands of connective tissue continuous with that in the wall of the podium or ampulla. The strands are sometimes

Table II

The affinities of echinoid groups as determined by podial and ampullar morphology. The position of the urechinids and pourtalesids was assigned on the basis of Hyman's (1959) report of one pore per podium. The group "irregularia" here excludes the urechinids and pourtalesids, as the "stirodonta" excludes the Stomopneustidae and Phymosomatidae, and the "camarodonta" excludes the Echinometridae and Parasaleniidae

I. One pore in body wall per podium
    Urechinidae
    Pourtalesiidae
II. Two pores in body wall per podium
    A. No septae in ampullae
        Echinothuroida
    B. Septae in ampullae
        1. Strands in aboral podia
            Cidaroida
            Stirodonta
            Irregularia
        2. Septae in podia
            a. Aboral podia without suckers
                Aulodonta
                Echinometridae
                Parasaleniidae
            b. Suckered aboral podia
                Phymosomatidae
                Stomopneustidae
                Camarodonta

arranged closely in rows, forming septae. Many, possibly all, septae and strands are covered with an epithelium. The strands and septae probably have a common point of origin in the ancestors of the cidarids. The podial septae probably originated in the condensation into a continuous septum of the linear arrangement of strands in the sensory podia of ancestral stirodonts. The podia morphology of the echinoids diverged with time, the most highly adapted respiratory podia being present in those forms burrowing in the relatively anaerobic substrate.

The morphology of echinoid podia are of taxonomic importance. The affinities of the echinoids as based solely on podial and ampullar morphology can be seen in Table II. The echinothurids have the simplest morphology of all regular echinoids. The echinothurids are not similar to the aulodonts as predicted by Durham and Melville (1957). If the Diadematidae and Pedinidae have converged during evolution as Philip (1965) suggested, they have converged in podial morphology as well as lantern structure, *etc.*

The podial morphology of the aulodonts is mid-way between that of the stirodonts and the camarodonts, not between the cidarids and stirodonts as Durham and Melville's (1957) classification would predict. It seems likely that the aulodonts diverged from the stirodont stock that originated the podial septum. From that same stirodont stock, a different, divergent line developed aboral, suckered tube-feet in the phymosomatids and stomopneustids, and then echinoid-type plates and a camarodont lantern in the temnopleurids. This implies that the short, blunt aboral podia of the echinometrids and parasalenids are "degenerate," while the tall, blunt aboral podia of aulodonts are "primitive." Mortensen's (1928–1951) classification most nearly predicts the podial affinities found.

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SUMMARY

1. The morphology of the podia and ampullae of thirty echinoids spread over 7 orders is described.
2. Four adaptations for respiration are found in the podia and ampullae of echinoids. The first is the separation of ciliary currents in the podium-ampulla lumen by two pores in the body wall, a septum in some podia, and the ampulla's flattened shape. The ampullae are crossed by septae.
3. The second respiratory adaptation is an increase in surface area of ampullae and podia by flattening. Cidarids, arbacids, and spatangoids further increase podial surface area by folding.
4. The ciliary currents inside and outside of ampullae and some podia move in a counter-current, increasing respiratory efficiency.
5. The fourth adaptation for respiration is that all podia having the first three adaptations are favorably positioned on the animal for respiration. Ampullae adapted for respiration serve podia adapted for respiration.
6. All strands and septae crossing echinoid podial or ampullar lumina contain strands of connective tissue.
7. Mortensen's (1928–1951) classification most nearly predicts the podial affinities found.

LITERATURE CITED

Bather, F. A., 1900. *The Echinoderms.* Pages 1–344 in R. Lankester, Ed., *A Treatise on on Zoology, Volume 3.* Adam and Charles Black, London.

Chadwick, H. C., 1900. *Echinus. Memoirs of the Liverpool Marine Biological Society,* **3**: 1–28.

Chesher, R. H., 1969. *The Systematics of Sympatric Species of West Indian Spatangoids: A Revision of the Genera Brissopsis, Plactholaenia, Paleopneustes, and Savinaster.* Studies in Tropical Oceanography No. 7, University of Miami Press, Miami, 168 pp.

Coleman, R., 1969. Ultrastructure of the tube foot wall of a regular echinoid, *Diadema antillarum* Philippi. *Z. Zellforsch. Mikrosk. Anat.,* **96**: 162–172.

Copeland, D. E., 1968. Fine structure of salt and water uptake in the land crab, *Gecaricinus lateralis. Amer. Zool.* **8**: 417–432.

Cuenot, L., 1948. Antomine, ethologie, et systematiques des echinoderme. Pages 1–363 in P. Grasse, Ed., *Traite de Zoology, Volume 11.* Masson, Paris.

Durham, J. W., and R. V. Melville, 1957. A classification of echinoids. *J. Palenotol.,* **31**: 242–272.

Durham, J. W., 1966. Classification. Pages 270–295 in R. C. Moore, Ed., *Treatise on Invertebrate Paleontology, part U, Echinodermata 3.* Geological Society of America, New York.

FARMANAFARMAIAN, A., 1959. Respiratory surface of the purple sea urchin (*Strongylocentrotus purpuratus*). *Doctoral dissertation, Stanford University*, California, 98 pp.

FARMANFARMAIAN, A., 1966. Respiratory physiology of echinoderms. Pages 245–266 in R. A. Boolootian, Ed., *Physiology of Echinodermata.* John Wiley, New York.

FARMANFARMAIAN, A., 1968. The controversial echinoid heart and hemal system- function effectiveness in respiratory exchange. *Comp. Biochem. Physiol.*, **24**: 855–863.

FENNER, D. H., 1971. A comparative study of the respiratory adaptations of the podia of echinoids. *Biol. Bull.*, **141**: 385–386.

GREY, F., 1954. *The Microtomist's Formulary and Guide.* The Blakiston Co., Inc., New York, 794 pp.

GURR, E., 1962. *Staining: Practical and Theoretical.* The William and Wilkins Company, Baltimore, 631 p.

HUGHES, G. M., AND A. V. GRIMSTONE, 1965. The fine structure of the secondary lamellae of the gills of *Gadus pollachius. Quart. J. Microscop. Sci.*, **106**: 343–353.

HUBSCHMAN, J. H., 1962. A simplified azan process well suited for crustacean tissue. *Stain Tech.*, **37**: 379–353.

HYMAN, L. H., 1955. *The Invertebrates: Volume IV Echinodermata.* McGraw-Hill Book Company, New York, 763 pp.

KAWAGUTI, S., 1964. Electron microscopic structures of the podial wall of an echinoid with special references to the nerve plexus and muscle. *Biol. J. Okayama Univ.*, **10**: 1–12.

KAWAGUTI, S., 1965. Electron microscopy on the ampulla of the echinoid. *Biol. J. Okayama Univ.*, **11**: 75–86.

LUDWIG, H. VON, 1904. *Die Seeigel.* Pages 967–1345 in H. C. Bronn, Ed., *Klassen und Ordnungen des Their-Reichs, Band II, Abtheilung 3, Buch 4.* Verlagshandlung, Leipzig.

MACBRIDE, E. W., 1909. *Echinodermata.* Pages 1–623 in W. F. Harmar, and A. E. Shipley, Eds., *Cambridge Natural History, Volume I.* Macmillan and Company, London.

MORTENSEN, T., 1928–1951. *A Monograph of the Echinoidea: Volumes I–V.* Milford, Oxford University Press, London, 4472 pp.

NICHOLS, D., 1959a. The histology and activities of the tube-feet and clavulae of *Echinocardium cordatum. Quart. J. Microscop. Sci.*, **100**: 73–87.

NICHOLS, D., 1959b. The histology and activities of the tube-feet of *Echinocyamus pusillus. Quart. J. Microscop. Sci.*, **100**: 539–555.

NICHOLS, D., 1961. A comparative histological study of the tube-feet of two regular echinoids. *Quart. J. Microscop. Sci.*, **102**: 157–180.

NICHOLS, D., 1966. Functional morphology of the water-vascular system. Pages 219–244 in R. A. Boolootian, Ed., *Physiology of Echinodermata.* John Wiley, New York.

PAUL, C. R. C., 1968. The morphology and function of dichoporite pore-structures in cystoids. *Palaeontology*, **11**: 697–730.

PHILIP, G. M., 1965. Classification of echinoids. *J. Paleontol.*, **39**: 45–62.

SCHAFFER, H., 1962. Die scutelliden des miozans von Osterreich und Ungarn. *Palentol. Z.*, **36**: 135–170.

STEEN, J. B., 1965. Comparative aspects of the respiratory gas exchange of sea urchins. *Acta Physiol. Scand.*, **63**: 164–170.

YOSHIDA, M., 1966. Photosensitivity. Pages 435–464 in R. A. Boolootian, Ed., *Physiology of Echinodermata.* John Wiley, New York.

Reference: *Biol. Bull.*, **145**: 340–351. (October, 1973)

REPRODUCTION TESTS: THE TOXICITY FOR *ARTEMIA* OF DERIVATIVES FROM NON-PERSISTENT PESTICIDES[1]

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The primary objective of the present study was to investigate the reproductive performance of a small crustacean after exposure to sublethal doses of the types of organic compounds occurring early in the degradation of non-persistent pesticides. In choosing the compounds to be tested, our guide was the United States Department of Interior monograph by Menzie (1969). Although the original toxicant may be converted within days or weeks, aromatic ring structures can persist. One transformation well established for sea-water is the hydrolysis of Sevin® to 1-naphthol (Stewart, Millemann and Breese, 1967). A more comprehensive study of the steps of biological degradation in the aquatic environment has employed river water and sewage (Aly and El-Dib, 1972).

Using the brine shrimp *Artemia salina* Leach as the assay organism, we conducted an exploratory screening experiment on nine different compounds. Three of these failed to reduce adult life span or depress fecundity at 10 parts per million (ppm): benzazimide and 3-hydroxy-methylbenzazimide from azinphosmethyl, and 3,5,6-tricholoro-2-pyridinol from Dursban®. This paper gives the results of a follow-up study of the 6 remaining agents listed on Table 1.

Complete reproductive records are summarized for adult *Artemia* of two different histories, (1) those removed to standard rearing conditions after only one day's exposure, and (2) those maturing a year later in treated mass populations reconstituted after routine over winter evaporation. Observations on the mass populations are also presented.

Materials and Methods

The stock of *Artemia* used for the present experiments was begun in 1957 from a mass hatching of thousands of California cysts. By 1971 when these experiments began, the population had 14 years of adaptation to laboratory conditions. Characterization of the reproductive performance of the stock is given in previous papers (Grosch, 1966, 1967). Since its origin the stock has over-wintered on shelves in windows with South West exposure in rooms maintained above 50° C at the Marine Biological Laboratory of Woods Hole, Massachusetts. Most important for the successful mass rearing of *Artemia* is my use of annual evaporation to eliminate the repressive influence of accumulated secretions and excretions. This occurs during the winter months. Annually in June cultures are reconstituted by adding

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TABLE I

The tested compounds listed in relation to the pesticides from which they can be derived

Name of pesticide	Chemical name	Derivative tested	Abbreviation used
Parathion	0,0-diethyl 0-(p-nitrophenyl) phosphorothioate	p-nitrophenol	p-N
Diazinon	0,0-diethyl 0-(2-isopropyl-4-methyl-6-pyrimidinyl) phosphorothioate	2-isopropyl-4-methyl-6-hydroxy-pyrimidine	Iso
Carbaryl (Sevin)	1-naphthyl-N-methylcarbamate	1-naphthol	1-N
		1,5-dihydroxy naphthalene	1,5-D
		1-naphthyl-hydroxy-methyl carbamate	C
Trichlorobenzene*	1,3,5-trichlorobenzene	1,3,5-trichloro-benzene	TCB

* Chlorinated benzene compounds have been used for termite control and also are derived in the degradation of several types of pesticides.

distilled water to the salts and cysts deposited during the gradual evaporation. Under these conditions, a 20-liter battery jar will show more than 2000 larvae and produce 1000 adults.

In 1971 when post-nauplial larvae had developed, 1-liter portions of the mass cultures were distributed to a number of subculture jars which were gradually brought up to 3 liters by small daily additions of filtered sea-water. As they matured, shrimp were distributed to provide 100 adults in each 3-liter subculture. Each compound tested was dissolved in 1 ml of acetone and stirred into one of the subcultures to provide a simple ecological system of volunteer algae, shrimp, and sea-water containing 10 ppm of the chemical. In our experience with *Artemia* (Grosch, 1967) as well as in general toxicology, acetone has proved to be relatively harmless (Moeschlin, 1965) and useful as an organic solvent.

In the preliminary screening, exposures up to 4 days were attempted and the solutions tested ranged downward from 100 ppm to 0.01 ppm. Above 10 ppm not all compounds were soluble. With those that were, deaths resulted even after short exposures. Exposures at 10 ppm for 2 days appeared to approach the early-death limit for several agents. On the other hand, decreases in reproductive performance were not always evident after exposures to 1 ppm. A compromise was to remove one group of adults for study after a day's exposure to 10 ppm, continue observation of the treated mass culture through the summer of 1971, and a year later study reproductive performance of a sample of adults from each population still viable.

Therefore, 24 hours after addition of a test compound, ten pairs were taken from each treated population. Each mated pair was placed in a separate jar containing

TABLE II

Adult life span and the components of their reproductive performance summarized as averages for samples of Artemia *comprised of 10 pairs subjected to each treatment of 10 ppm for 24 hours in 1971. Standard errors were calculated for all means but are not listed in some categories to conserve space. A basis for recognizing 2 classes is given in the text*

Chemical tested	Survival of adults (days)		Number of broods	Total no. of zygotes	Cysts (%)	Cysts hatched (%)	Larval survival (%)	Sex ratio no. males/ no. females	Adaptive value
	Males	Females							
None	49.6 ± 4.0	50.0 ± 5.0	11.3 ± 1.4	1828	29.0	46 ± 5	76.3 ± 5.0	0.91	
Acetone	47.6 ± 4.2	50.1 ± 5.5	11.8 ± 1.6	1884	30.6	48 ± 7	75.6 ± 4.7	0.94	1.00
Class I									
p-nitrophenol	37.7 ± 5.4	34.8 ± 5.1	5.7 ± 1.8	1031	33.8	44 ± 6	72.4 ± 5.6	0.86	0.48
2-isopropyl-4-methyl-6-hydroxy pyrimidine	37.3 ± 2.7	33.6 ± 2.1	8.2 ± 0.8	1060	3.7	61 ± 9	70.2 ± 8.6	0.79	0.61
1-naphthol	30.9 ± 3.4	31.3 ± 2.4	6.3 ± 0.9	1062	11.6	50 ± 11	84.6 ± 6.8	0.97	0.70
Class II									
1,5-dihydroxy naphthalene	25.2 ± 2.5	22.1 ± 1.9	4.9 ± 0.7	463	21.5	42 ± 8	46.2 ± 12.9	0.95	0.16
1-naphthyl hydroxymethyl carbamate	23.3 ± 9.6	33.6 ± 9.2	7.2 ± 1.9	694	2.8	80 ± 12	52.6 ± 7.9	0.54	0.38
1,3,5-trichloro-benzene	44.2 ± 3.8	37.6 ± 4.2	5.3 ± 0.8	456	11.4	18 ± 10	30.3 ± 11.5	0.82	0.11

500 ml of a standard brine solution (Grosch, 1967). Subsequently each pair was fed 0.5 ml of yeast suspension daily. Before each feeding, zygotes were removed and counted. Larvae produced viviparously were transferred by groups into separate rearing jars, maintained until maturity in standard brine. Cysts were filtered, dried and resuspended in sea-water to determine the proportion from which larvae could emerge. Both the pair-mating jars and the rearing jars were kept under constant fluorescent light which holds the water temperature between 26°–28° C.

After 10 pairs had been removed for life-time reproductive performance studies, the 3-liter treatment jars with 80 adults were shelved. Jars showing live *Artemia* were fed daily with yeast suspension. In all jars the water level was maintained until September 1 when the annual evaporation phase began.

In early June of 1972 cultures in the treatment jars were reconstituted by adding distilled water to their contents. In jars where adults matured, again pairs were set up in individual jars of brine to obtain data on life span and reproductive performance.

When summarized data were tested statistically, t values were calculated from the ratio of the mean difference to the variance of the difference. Upon comparison with the standard *t* table the 0.05 probability level was taken as significant and the 0.01 level as highly significant evidence against the null hypothesis.

A mathematical approach to fitness which will be shown of limited predictive utility for *Artemia* populations is the "adaptive value." In its simplest form, this

TABLE III

Adult life span and the components of their reproductive performance summarized as averages for samples of the Artemia *adults matured in the mass populations reconstituted in 1972 from an overwintering evaporation phase. Standard errors were calculated for all means but are not listed in some categories to conserve space*

Chemical tested	1972 Generation tested	Adult life span (days)		Number of broods	Total no. of zygotes	Cysts (%)	Cysts hatched (%)	Larval survival (%)	Sex ratio no. males/no. females	Adaptive value
		Males	Females							
Acetone control	1st	41.1 ± 4.3	40.0 ± 4.9	10.0 ± 1.4	1221	31.1	58 ± 5	70.2 ± 6.1	0.96	1.00
p-nitrophenol	1st	33.3 ± 3.5	30.5 ± 4.0	6.3 ± 1.0	1239	33.1	59 ± 4	65.2 ± 8.4	0.96	0.93
2-isopropyl-4-methyl-6-hydroxy pyrimidine	1st*	12.0	24.0	4.0	365	86.8	67 ± 8	75.2 ± 8.7	0.82	0.26
	2nd	36.0 ± 2.2	32.4 ± 1.0	8.0 ± 0.8	1148	15.4	60 ± 4	68.2 ± 7.7	0.99	0.97
1,5-dihydroxy naphthalene	1st	26.3 ± 4.6	21.6 ± 1.7	3.1 ± 0.4	523	70.2	76 ± 3	64.1 ± 6.6	0.92	0.37

* The initial 1972 generation consisted of 3 adult females and 1 male. All other data tabulated come from 10 pairs from each population.

measurement consists of dividing the average number of matured offspring per pair by the average number of matured offspring per control pair. Often for simple comparisons one of the strains is arbitrarily designated as having a fitness of 1.00 (Strickberger, 1968). For our purposes the acetone control was so designated. The adaptive values obtained (A.V.) from pair mating tests are given on Tables II and III and evaluated in the Discussion.

RESULTS

1971 pair mating tests

These results are summarized in Table II. A trend toward decreased life span was revealed for all treated adults. While a statistically significant difference from the control mean cannot be established for males exposed to 1,3,5-trichlorobenzene "TCB," the premise of shortened life spans can be accepted for all other treated groups. When the difference between means is 12 days, *t* values achieve the 0.05 level; when the difference is 15 or more days, *t* exceeds the 0.01 level. In general, the incidence of death was distributed so equally between the sexes that when a male died his female could be supplied from the same test group with a male that had recently lost his mate. Assuring females of mates was possible even in the 1-naphthyl hydroxymethyl carbamate "C" tests when males tended to die earlier than females.

The average total number of broods per pair was decreased by every agent. Statistically when the difference from the control mean amounts to 5 or more broods, *t* values exceed that for $t_{0.995}$. Even when the difference is only 3.1 (11.3–8.2) *t* approached the 0.5 level.

In most of the experimental groups the deficit could be a consequence of the decrease in life span. With successive broods appearing at 3 to 4 day intervals, shortening the female life span by 15 days results in 4 or 5 broods fewer than the control pairs. In addition, 1,5-dihydroxynaphthalene "1,5-D" and TCB caused a delay in the appearance of the first brood for more than a week. Thus, an addi-

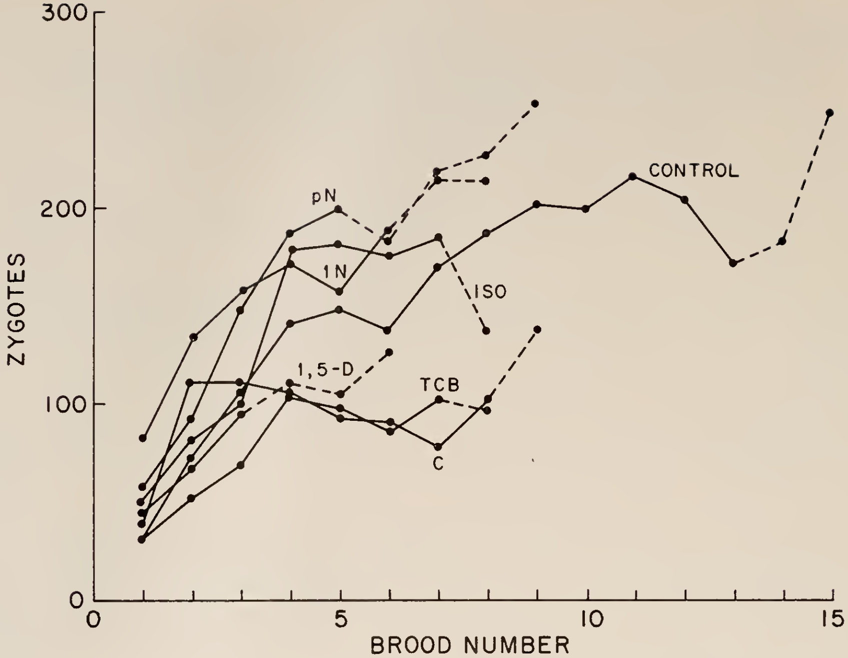

FIGURE 1. The average brood size plotted in the sequence of its production by pairs withdrawn after 24 hours from the populations of *Artemia* subjected to degradation products of pesticides in 1971. Abbreviations are: pN, p-nitrophenol; 1 N, 1 naphthol; ISO, 2-isopropyl-4-methyl-6-hydroxypyrimidine; 1,5-D, 1–5-dihydroxynaphthalene; C, 1-naphthyl hydroxymethyl carbamate; TCB, 1.3,5-trichlorobenzene. The broken line indicates that less half of the pairs remain alive.

tional two or more broods were subtracted from a potential life time total curtailed by a shortened life span. During the sterile periods there was no reluctance to mate. Direct observations of pair activities ruled out a change in mating behavior.

A decrease in the number of broods was accompanied by a decrease of the average total number of zygotes produced. Such an influence explains the three deficiencies of about 800 zygotes giving totals of slightly more than 1000 zygotes, but it does not explain the other three cases where life time totals amounted to less than a third of the control value. Plotting the average size of each brood in the sequence of its production (Fig. 1) reveals two classes of response to the chemicals tested. In one class three of the experimental groups produced offspring at a rate equal to or exceeding that of the controls but they were not living long enough to produce a series of broods in the 200 offspring category attained by the controls. On the other hand, the 3 poorly performing groups of Table II are shown in Figure 1 to have had a trend to brood size smaller than controls. This trend becomes evident by the third brood. Thus, in a second class of experimental shrimp certain chemicals decreased the number of zygotes per brood. When averages are calculated the three groups of the second class averaged under 100 zygotes per brood, respectively 94.5, 96.4 and 86.0 zygotes per brood.

Further damage from the three more deleterious compounds was revealed in the lower larval survival (Table II), a result especially notable in *Artemia* where relaxation of crowding is ordinarily accompanied by improved survival to maturity. In contrast, the offspring from controls and from the three less deleterious experimental groups showed an excellent proportion of larvae surviving to maturity. A predominance of the digametic sex (females) among matured larvae, shown by sex ratios below 1.00, rules out the action of significant numbers of induced recessive sex-linked lethal mutations.

In the tests summarized in Table II the treated females produced too few cysts to provide reliable evidence on cyst hatchability. In particular the "C" test provided almost no cysts and a decrease in oviparity was noted for 4 of the other 5 experimental groups.

Treated populations in 3-liter jars

Three of the populations survived less than a week because all the adults remaining in the mass rearing jars died without producing young of either type. These were the jars receiving either 1-naphthol "1-N," 1-naphthyl-hydroxymethyl-carbamate "C," or 1,3,5-trichlorobenzene "TCB." Although cysts were recovered from the 1-N jar, none contained viable embryos. In the other three treated populations adults remained alive for the entire summer. Despite recurrent pairing and copulation, viviparity was rare, and post-naupliar stages never seen. Cysts accumulated at the high water line only during the first few days.

Despite the water's persistent intense yellow color, mature adults of the p-nitrophenol "p-N" culture were able to survive the entire summer of 1971. At reconstitution of the evaporated culture in June, 1972, many yellow crystals were present among the salts at the bottom of the jar. When the salts dissolved, the brine again had a definite yellow color. Nevertheless there was a massive hatch of cysts from the sides of the jar, and more than 100 adults matured. In turn these produced viviparous young during 1972. Several hundred shrimp from these broods survived to maturity.

Upon rehydration in 1972 of the culture given 2-isopropyl-4-methyl-6-hydroxypyrimidine "Iso" there was a copious hatching of cysts but only 4 females and 1 male survived to maturity. After producing a first brood of larvae, these 5 adults were removed from the mass culture jar for a study of their reproductive performance. By July 20, 1972 the larvae remaining in the 3-liter jar had given rise to more than 100 adults. Presumably removal of the adults from the jar expedited maturation of a second summer generation. Of these, the reproductive performance of 10 pair matings was studied in individual 500 ml jars.

After receiving 1,5-dihydroxynaphthalene "1,5-D" the 3-liter culture developed a dirty yellow cloudiness during the first 24 hours and an odor of naphthalene which persisted for over a week. Subsequently finely divided black debris settled out of the culture in the course of the summer. Some of this was identifiable as dead larvae. At culture reactivation in 1972 numerous larvae hatched from the cyst layer at the high water mark. Although survival to maturity seemed poor, more than 100 reproducing adults comprised the population by the end of August.

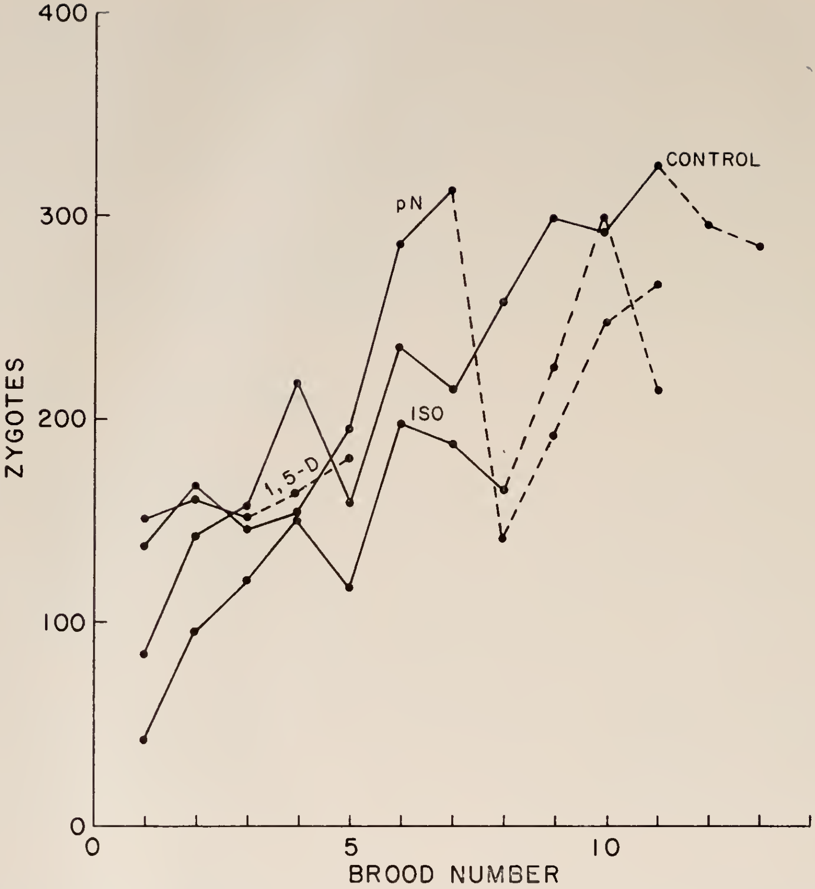

FIGURE 2. The average brood size plotted in the sequence of its production by pairs from the populations of *Artemia* surviving the evaporation-reconstitution cycle of 1971 to 1972. Abbreviations are: pN, p-nitrophenol; ISO, 2-isopropyl-4-methyl-6-hydroxypyrimidine; 1,5,-D,1,5-dihydroxynaphthalene. The broken line indicates that less than half of the pairs remain alive.

1972 pair mating tests

Results are summarized in Table III for the jar populations that survived to provide shrimp for testing a year after treatment. Adults from treated populations tended to die earlier than those from the control, but in 1972 the mean life span of controls was about a week shorter than in 1971. Accordingly, the differences between means are not large, and t tests reveal significant decreases only for males and females of the 1,5-D group. The first IMH generation of only 1 male and 3 females was inadequate for such analysis.

A ranking of the average number of broods is concomitant with a ranking of female life spans. The t value for 1,5-D exceeds that for $t_{0.995}$. Even the t value for p-N number of broods exceeds the 0.05 level. On the other hand, the p-N pairs had an average of total zygotes higher than that of the year's controls.

An appreciable proportion of zygotes were encysted for all groups tested, and the average percentage hatching exceeded the control value in all experimental groups. In 1972 the survival of larvae to maturity approached the control level in data from pair tests. The relaxation of crowding in the few broods from the 3 females available as the first 1972 generation of the IMH treated population is reflected in the 75.2% average survival of larvae. This exceeds the control value but not to a degree considered statistically significant. The sex ratios among the maturing offspring were all under 1.00 but consistently high. For all groups tested, first broods appeared without delay, and subsequently, broods were deposited every 3 to 4 days with regularity.

Plotting the average size of each brood in the sequence of its production (Fig. 2) reveals that pairs from the p-N populations exceeded the 200 offspring per brood level after the fifth brood. This was accomplished by a higher rate of increase in brood size than that of controls from the 4th to 7th broods deposited. On the other hand, in comparison with the controls, smaller broods were produced by the females of the Iso treated population, although the increase in brood size occurred at about the same rate as the controls. In contrast, the size of the broods deposited by females from the 1,5-D population was adequate for early broods. However, most of the shrimp from this population died after producing only 3 broods.

Discussion

The pesticides of concern may enter coastal waters not only from their use in forestry and agriculture, but also through attempts to control estuarine shellfish predators (Karinen, Lamberton, Stewart and Terriere, 1967). An incentive for this research was the disquieting thought that the initial actions of environmental degradation may not provide biologically inert products from the molecules of so-called non-persistent pesticides. In addition, one category of derivatives, the naphthalene compounds, can escape to the environment from the manufacture of dyes, synthetic resins, explosives, lubricants, and motor fuels.

A wide variety of effects from pesticides on non-target organisms has been reviewed by Pimentel (1971), but with crustacea attention has tended to focus on the determination of immobilization and lethal doses. A notable contribution to crustacean toxicology has been the development of the *Daphnia magna* bioassay (Parker, 1965; Frear and Boyd, 1967). However, here too, emphasis has not been given to reproductive performance. A special feature of the present study is the determination of the number and fate of *all* zygotes in the *sequence* of their production. The mammalian design of the reproduction test which employs one selected litter or a group of several litters (Fitzhugh, 1968) is inadequate for an organism like the shrimp where size is indeterminate and brood size increases with the increasing size and age of the mother. Within this framework it seems important to distinguish between toxic agents that may merely cause maternal debility (Class I) and those which include a more direct attack upon reproduction (Class II). Postulated to explain the Class II data of Table II is the vulnerability of dividing cells. On this basis, damage is expected in the stem cell component of the *Artemia* gonads and to the cells of the cleaving embryo. Embryos attain the blastula stage within the female before a choice between encystment or viviparity is made for them (Lochhead, 1941).

In mammalian toxicology, deficiencies in proliferating cell populations are caused by compounds chemically related to those of our Class II. Benzene and its derivatives including naphthalene are known to cause bone marrow depletion with associated leukopenia and anemia (Moeschlin, 1965). Less familiar are the cytological investigations with plant meristems which demonstrated that members of the halogenated naphthalene series inhibit mitosis in low doses and are cytotoxic at higher concentrations (Gavaudan and Gavaudan, 1940; Levan and Ostergren, 1943). However, until now none of the naphthalene and carbamate compounds of Table I seem to have received cytogenetic consideration. Most recently we have obtained data on the fecundity and fertility of *Habrobracon* (Hymenoptera: Braconidae) females following exposure to the three most effective compounds (Grosch and Hoffman, 1973). In this wasp the single series of oogenetic stages per ovariole make possible an exact identification of the most sensitive types of cells. These proved to be the oogonia engaged in the five mitotic divisions necessary to form the folliculate nests, each containing an oocyte and 31 nurse cells (Grosch, 1965). Cells irrevocably damaged at this point never form oocytes. Others give rise to oocytes but their embryos fail to complete the nuclear cleavage divisions. These braconid results are strikingly parallel to those for *Artemia,* but a complete correlation of ovarian cell responses between the two quite different Arthropods will require experiments in which only female *Artemia* are exposed to the naphthalene compounds. On the other hand, there is a definite difference in somatic sensitivity between the adult insect and the adult shrimp. The braconid is relatively tolerant because its somatic tissues are no longer mitotic, but the female shrimp molts before each brood is produced (Lochhead, 1941). Since adult males also continue to molt and grow throughout adulthood, the presence of cell divisions may be presumed. Thus, epidermal mitoses associated with the periodic molting of both sexes may help to explain the particularly short adult life spans following exposure to naphthalene compounds.

The biodegradable pesticides providing the derivatives tested have received more attention. In chickens, ingested carbaryl decreased egg hatchability and induced congenital malformations (Ghadiri *et al.,* 1967). However, at doses 1000 times the allowable level of the pesticides, carbaryl produced terata only in guinea pigs, not in hamsters and rats (Robens, 1969). Diazinon was non-teratogenic in small laboratory animals (Robens, 1969). In a 3 generation reproductive study (Collins, Hanson and Keeler, 1971), rat and gerbil fertility was impaired by 10,000 ppm of carbaryl (Sevin®) in the food, but a more recent summary of all the investigations using dietary doses no higher than 2000 ppm concluded that there are no reproductive effects in rodents at realistic dose levels (Weil, Woodside, Carpenter, and Smyth, 1972). In mammals and insects carbamate and naphthalene molecules are metabolized by the microsomal enzymes (Parke, 1968). A survey of marine forms showed Sevin more toxic to larval and adult crustaceans than to molluscs and fish (Stewart, Millemann and Breese, 1967), but no data was obtained on reproductive performance. Subsequently flaccid ovaries were found to be in a resorptive state for the infecund minnows resulting from the highest dose level of a series of 9 month exposures to Sevin (Carlson, 1972).

Some of the consequences of exposing shrimp populations to carbamate derivatives is provided by observations reported here. Generally, fitness is defined as the relative capacity for leaving offspring that attain reproductive age (Mettler and Gregg, 1969). To accomplish this, *Artemia* along with many other organisms have adopted the reproductive strategy of investing their energy in gamete production. The loss of a considerable proportion of potential offspring can be serious. A reduction of the number of zygotes to $\frac{1}{3}$ or less than the control average, as caused by 3 of the tested compounds, implies impending population collapse. In long term studies of irradiated populations, more than half of the normal reproductive capacity constituted a reserve necessary for buffering environmental changes (Grosch, 1966).

In addition to quantity, the quality or type of zygote is significant for *Artemia* population survival. Shunting offspring into a dormant encysted state is the usual *Artemia* response to unfavorable external factors. By interfering with this response most of the chemicals added to jar cultures in 1971 had the effect of increasing the vulnerability of shrimp to their environment. Furthermore, response of the shrimp could not be predicted from the appearance of the water in which they lived. The two visibly contaminated cultures have survived while others with clear water became exinct.

The proportion of zygotes encysted may be the most important index to strain survival, provided some of the embryos are viable. Since cyst production is a prompt response important for surviving an environmental change, it was not surprising to observe a significant shedding of cysts within a day or two after a chemical contamination of a jar population. At the time this important component of cysts was produced, the adults of the entire population were equivalent to those removed for pair mating tests. Subsequently, the reproductive capacities of the mass population and the pairs removed to uncontaminated water diverged.

Although the 1971 A.V.'s serve as a basis for comparing the reproductive performance of pairs removed from the treated 3 liter culture after 24 hours, they are inadequate for predicting the future of the population left behind to experience a longer exposure. Thus, a high A.V. of 0.70 for pairs exposed to 1-naphthol is inapplicable to the performance of adults that died without leaving viable cysts in the 3-liter population jar. In contrast, the 1,5-D treated population persisted to 1972 despite a low 1971 A.V. from the pair matings. In this case the moderate proportion of encysted zygotes obtained in the pair mating tests presaged an adequate deposit of viable cysts on the walls of the overwintering population jar. The outstanding example of an impressively persistent population was the one exposed to p-N. Its 1971 A.V. was a low 0.48 but the proportion of zygotes encysted, 33.8%, was higher than the control values.

Following a cycle of evaporation and reconstitution of the overwintering jars, the 1972 A.V.'s for pair matings should adequately represent the performance of the shrimp from the surviving populations. Although none have reached the control level, all show improvement over the 1971 A.V.'s. With Iso, it was possible to show impressive improvement in one generation during the summer of 1972, from an A.V. of 0.26 for the three females and 1 male constituting the first generation to the 0.97 for the pairs used to sample the second generation. Equally important was the increase in the proportion of cysts deposited. Notably the strain with the lowest A.V. (1,5-D) has responded to stress by producing a high proportion of cysts with good hatchability.

The azinphosmethyl derivatives were gifts from Chemogro Chemical Company, Kansas City, Missouri; the Dursban® derivative from Dow Chemical Company, Midland, Michigan; the carbaryl derivatives from Union Carbide Experiment Station, Clayton, North Carolina; and the diazinon derivative from Geigy Chemical Company, Ardley, New York.

The industrious attention of Janet Guthrie to preliminary experiments must be acknowledged. She was a January plan student from the University of Delaware.

SUMMARY

(1) This paper reports (a) the differential extinction and persistence of 3-liter mass populations following the addition of 10 ppm of 6 derivatives of biodegradable carbamate and organophosphorus pesticides, (b) the reproductive performance of *Artemia* pairs removed from the 6 treated populations after 24 hours, and (c) reproduction tests of pairs from the populations surviving an overwintering evaporation cycle.

(2) Life span was decreased to some degree for adults treated with any of the 6 tested derivatives of "degradable" pesticide. A concomitant decrease in life time totals of offspring can explain the results for 3 of the compounds.

(3) The reproductive performance is further curtailed by cytogenetic destructive action of naphthalene and carbamate types of compounds on gametes and zygotes. The implied mode of action is on dividing cells. Furthermore, related naphthalene compounds are spindle poisons.

(4) An adequate "standing crop" of adults is no assurance of survival of the population. More significant is the presence of either live larvae or viable cycts. The latter are especially important if the culture is subjected to periodic evaporation as is typical of many natural salterns.

(5) Appreciable improvement in the components of reproductive fitness was observed for surviving populations a year after the initial treatment.

LITERATURE CITED

ALY, O. M., AND M. A. EL-DIB, 1972. Studies of the persistence of some carbamate insecticides in the aquatic environment. *Advan. Chem.*, Series **3**: 210–243.

CARLSON, A. R., 1972. Effects of long-term exposures to carbaryl (Sevin) on survival, growth, and reproduction of the fathead minnow (*Pimephales promelas*). *J. Fish. Res. Board Can.*, **29**: 583–587.

COLLINS, T. F. X., W. H. HANSEN AND H. V. KEELER, 1971. The effect of carbaryl (Sevin) on reproduction of the rat and the gerbil. *Toxicol. Appl. Pharmacol.*, **19**: 202–216.

FREAR, D. E. H., AND J. E. BOYD, 1967. Use of *Daphina magna* for the microbioassay of pesticides. I. Development of standardized techniques for rearing *Daphnia* and preparation of dosage-mortality curves for pesticides. *J. Econ. Entomol.*, **60**: 1228–1236.

FITZHUGH, O. G., 1968. Reproductive Tests. Pages 75–85 in N. Boyland and R. Goulding, Eds., *Modern Trends in Toxicology*, Vol. I. Butterworths, London.

GAVAUDAN, P., AND N. GAVAUDAN, 1940. Action sur la caryocinèse, la cytodiécese, et la morphogénèse des végétaux de quelques dérivés d'hydrocarbures cycliques. *C. R. Soc. Biol. Paris*, **133**: 346–352.

GHADIRI, M., D. A. GREENWOOD AND W. BINNS, 1967. Feeding of malathion and carbaryl to laying hens and roosters. *Toxicol. Appl. Pharmacol.,* **10**: 392.

GROSCH, D. S., 1965. Biological effects of radiations. *Bios,* **36**: 55–62.

GROSCH, D. S., 1966. The reproductive capacity of *Artemia* subjected to successive contaminations of radiophosphorus. *Biol. Bull.,* **131**: 261–271.

GROSCH, D. S., 1967. Poisoning with DDT: Effect on reproductive performance of *Artemia. Science,* **155**: 592–593.

GROSCH, D. S., AND A. C. HOFFMAN, 1973. The vulnerability of specific cells in the oogenetic sequence of *Bracon hebetor* Say to some degradation products of carbamate pesticides. *Environmental Entomology,* in press.

KARINEN, J. F., J. G. LAMBERTON, N. E. STEWART AND L. C. TERRIERE, 1967. Persistence of carbaryl in the marine estuarine environment. Chemical and biological stability in aquarium systems. *J. Agr. Food Chem.,* **15**: 148–156.

LEVAN, A., AND G. OSTERGREN, 1943. The mechanism of c-mitotic action. Observations on the naphthalene series. *Hereditas,* **29**: 381–443.

LOCHHEAD, J. H., 1941. *Artemia,* the "brine shrimp." *Turtox News,* **19**: 41–45.

MENZIE, C. M., 1969. *Metabolism of Pesticides.* U. S. Department of Interior, Bureau of Sport Fisheries and Wildlife, Special Scientific Report Number 127, Washington, D. C.

METTLER, L. E., AND T. G. GREGG, 1969. *Population Genetics and Evolution.* Prentice-Hall, Inc., Englewood Cliffs, New Jersey, 212 pp.

MOESCHLIN, S., 1965. *Poisoning, Diagnosis and Treatment.* Grune and Stratton, New York, 707 pp.

PARKE, D. V., 1968. *The Biochemistry of Foreign Compounds.* Pergamon Press, Oxford, England, 269 pp.

PARKER, B. L., 1965. A carbamate bioassay technique and improved rearing procedure for *Daphnia magna. Diss. Abstr.,* **26**: 2944.

PIMENTEL, D., 1971. *Ecological Effects of Pesticides on Non-Target Species.* Office of Science and Technology, U. S. Gov. Printing Office, Washington, D. C., 220 pp.

ROBENS, J. F., 1969. Teratologic studies of carbaryl, diazinon, norea, disulfiram and thiram in small laboratory animals. *Toxicol. Appl. Pharmacol.,* **15**: 152–163.

STEWART, N. E., R. E. MILLEMANN AND W. P. BREESE, 1967. Acute toxicity of the insecticide Sevin® and its hydrolytic product 1-naphthol to some marine organisms. *Amer. Fish. Soc. Trans.,* **96**: 25–30.

STRICKBERGER, M. W., 1968. *Genetics.* Macmillan Company, New York, 868 pp.

WEIL, C. S., M. D. WOODSIDE, C. P. CARPENTER AND H. F. SMYTH, JR., 1972. Current status of tests of carbaryl for reproductive and teratogenic effect. *Toxicol. Appl. Pharmacol.,* **21**: 390–404.

Reference: *Biol. Bull.*, **145**: 352–359. (October, 1973)

PRIMITIVE NERVOUS SYSTEMS. A SENSORY NERVE-NET IN THE POLYCLAD FLATWORM *NOTOPLANA ACTICOLA*

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The nervous system of polyclad flatworms is comprised of a number of nerve tracts which radiate outwards from an anterior ganglionic mass often called the brain. These nerve tracts branch and anastomose repeatedly to form a network of strands. Two such networks have been recognized, (1) a ventral network of coarse nerves with a meshwork of finer fibers between the large strands and (2) a dorsal network of fine fibers (Hadenfeldt, 1928). Similar arrangements are found in molluscs and other invertebrates (Bullock and Horridge, 1965) but are generally less extensive. The network resembles the nerve-nets of coelenterates and echinoderms where there is diffuse conduction and information can be passed around cuts and obstructions in the nervous system. This kind of conduction has not been demonstrated in flatworms (Gruber and Ewer, 1962). On the contrary, in fact only discrete non-random conducting pathways have been demonstrated in this group. This is quite puzzling because the anatomical arrangement suggests a diffusely conducting system. Bullock and Horridge (1965) differentiate between a nerve-net and nerve plexus by considering the former as possessing diffuse conducting properties and the latter as an anatomical arrangement. The previously described discrete pathways in polyclad flatworms was surprising and the functional significance of the plexiform arrangement in this group is not clear (Horridge, 1968).

The physiological organization of polyclad nervous systems is of considerable importance from an evolutionary point of view. Polyclads are one order of platyhelminthes with clear affinities to the other major protostomous coelomates (*i.e.*, molluscs, annelids and arthropods) and are among the most primitive of these protostomes. Anatomically the nervous system is intermediate between that of coelenterates and the other protostomes, but the relationships between these groups is still controversial (Hadzi, 1963). If the flatworm nerve plexus possessed properties similar to those of the coelenterate systems then their intermediate position would be further substantiated.

The flatworm brain has considerable complexity (Best and Noel, 1969; Morita and Best, 1966; Turner, 1946) and early workers (Moore, 1923; Olmsted, 1922) demonstrated its importance for coordination of locomotory activity. Nothing is known, however, of the initiation of locomotory activity. This paper is concerned with the initiation of locomotion in the polyclad, *Notoplana acticola*, and the way that the nerve plexus transmits information to the brain. The observations made suggest that these creatures possess a sensory nerve-net.

METHODS

Animals

Mature specimens of *Notoplana acticola*, collected under rocks at Corona del Mar in Southern California were maintained in shallow plastic dishes of sea water at room temperature, approximately 20° C. Water was changed every other day. Animals were fed adult frozen brine shrimp and were maintained in good condition for over a month.

Recordings

One of the major difficulties encountered in utilizing polyclads is their fragility. It is very difficult to attach recording devices as preparations tend to disintegrate where pressure is applied. They cannot be pinned down for dissection as the body wall tears free from the pins. The only narcotizing agent found successful was 0.36 M $MgCl_2$, but the animals tend to disintegrate when returned to fresh sea water. Consequently most data was obtained by direct observation. In only one case was a force transducer (Statham Gold Cell) successfully attached to the animal for longer than a few minutes. In this instance the transducer was held in place by attaching it to a suction electrode on the body wall. The transducer was used to measure tension of the longitudinal body musculature. Permanent records of locomotory activity were made photographically.

Stimulation

Negative going square pulses were delivered from a Grass S5 stimulator through tygon-tubing suction-electrodes applied to the dorsal surface of an animal. The electrodes remained in place for only a few minutes before the tissue under them disintegrated. Mechanical stimuli were delivered by pricking the worm with a fine (#000) insect pin.

Observations were made in 100 mm petri dishes which had a thin layer of two per cent agar on the bottom and filled with sea water. The agar acted as a cushion against occasional dragging of the animal on the substrate when it was pricked. Animals were placed in the dish for several hours before use.

Cuts were made through the body of the animal with a sharp scalpel on the day prior to use. On the observation day the cuts had started to heal but the opposing cut edges were not yet rejoined. The relationship of cuts to nerve cords was verified by staining the nervous system with the indoxyl acetate method for general esterases developed by Halton and Jennings (1964). All experiments were repeated at least ten times unless otherwise stated.

RESULTS

Ditaxic locomotion

Normal escape movements of *N. acticola* consist of alternate waves of extension and contraction. The locomotory wave begins at the anterior of the animal and passes posteriorly along the length of the body, with left and right sides of the body being out of phase with each other. The extent of movement in the front portion of the body is more vigorous than that at the rear. This kind of movement is called ditaxic locomotion. In another polyclad, *Planocera*, the brain is necessary for ditaxic locomotion (Gruber and Ewer, 1962) and if it is excised only that part of the animal directly stimulated will contract. Therefore, the initiation of ditaxic locomotion can be used to indicate that sensory information must have reached the brain.

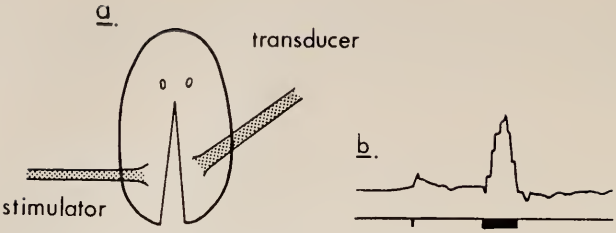

FIGURE 1. (a) A partially bisected flatworm with suction electrodes. (b) Response to a single stimulus and a train of stimuli. Intensity of each stimulus was 10 V and duration was 10 msec.

Responses to mechanical stimuli

The initial response to a pin prick at the posterior end of an animal is a small local contraction in the vicinity of the pin. If the worm is moving slowly the initial response is followed by increased anterior extension and locomotory rates. A stationary animal does not usually start to move unless the stimulus is repeated. A second jab within a few seconds of the first causes the animal to extend its anterior margin on one side and move away. Sometimes 3 or 4 pricks may be necessary to accomplish this.

Responses to electrical stimuli

Electrical stimuli do not elicit ditaxic locomotion. If they are applied between a single electrode on the posterior part of the animal and a ground in the surrounding water only localized twitches are produced, no matter how many stimuli are given. Similarly, shocks applied between two widely separated electrodes may produce considerable contraction between the electrodes without causing the animal to move. Electrical stimulation does cause a certain amount of propagated activity. In *Planocera* we showed that stimuli could be conducted from one side of the body to the other provided the brain was intact (Koopowitz and Ewer, 1970). When a specimen of *Notoplana* was split up the midline from the posterior margin to just behind the brain, electrical stimulation of one side also caused contractions to occur on both sides of the animal. In one preparation it was possible to measure the tension on the one side while stimulating the other side electrically (Fig. 1). Although mechanical stimulation can evoke ditaxis, electrical stimulation does not. Neither single nor multiple stimuli produce a response other than longitudinal contractions. This data indicates that acivity is promulgated from the one side of the animal to the other, probably through the brain.

Decerebrate animals

The brain is necessary to initiate locomotion. Animals from which the brain had been removed did not respond to mechanical stimulation. Instead they produced local twitches, reminiscent of those elicited by electrical stimulation.

Lesions posterior to the brain

Stimulating an animal behind a cut, which is posterior to the brain and has severed both of the major longitudinal nerve cords, still initiates ditaxic locomotion

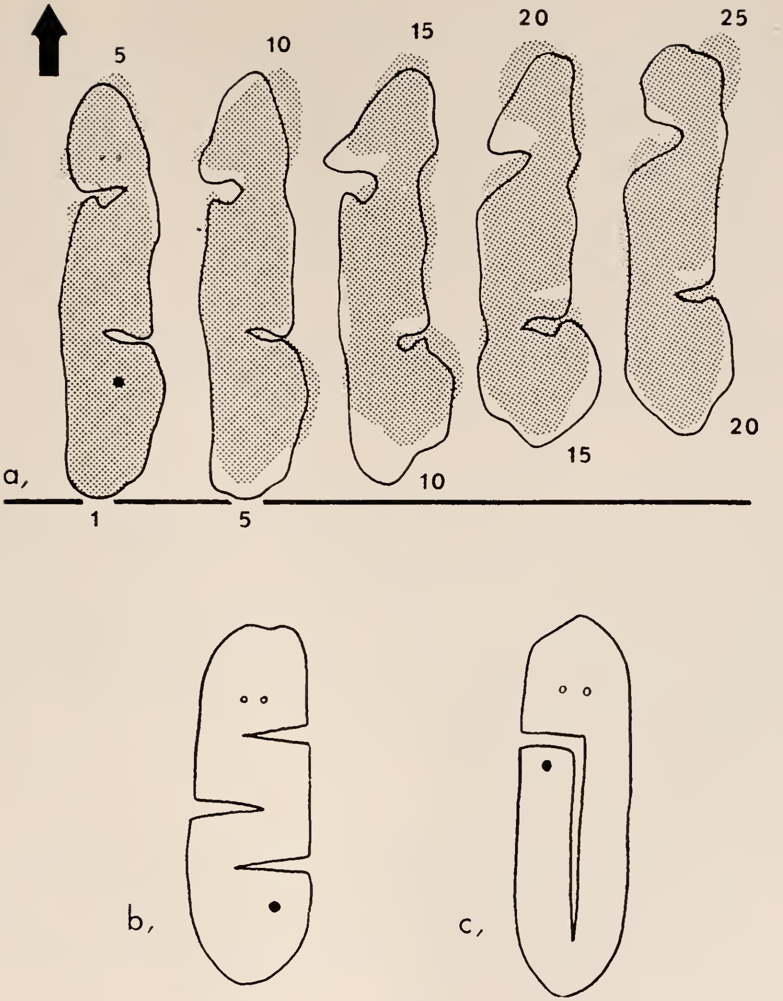

FIGURE 2. (a) Tracings of the first movements in ditaxic locomotion. This particular animal had two cuts which severed the longitudinal cords. Traced from a series of ciné film frames. Image with the solid contour represents the frame numbered at the bottom while the dotted area is the animal's position five frames later. Film speed was 16 frames per second. (b) Diagram shows the position of three interdigitating cuts through the animal's body; (c) position of cuts made to demonstrate posterior propagation of the stimulus. Solid dot is site of stimulation.

and the portion anterior to the cut is used for ditaxis. The part behind the cut does not appear to be involved with motor activity, on that side of the body.

It is also possible to initiate locomotion with mechanical stimuli delivered behind the most posterior of two interdigitating transverse cuts. Figure 2a illustrates the response evolved from a preparation in which the incisions were from

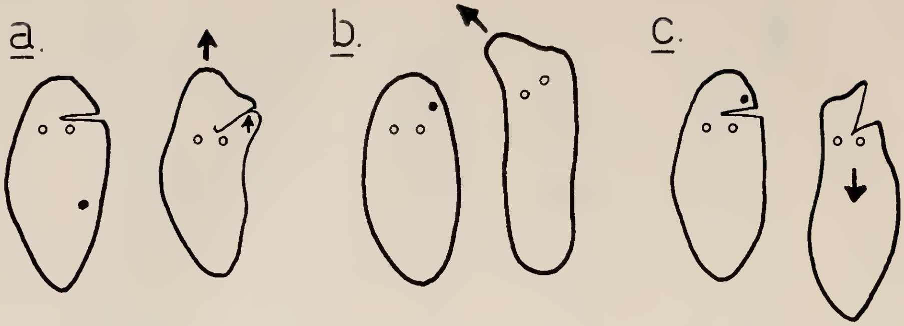

FIGURE 3. (a) The initial movement following posterior stimulation with an anterior cut. (b) Normal avoidance response to an anterior stimulus. (c) Avoidance response to an anterior stimulus after an anterior cut. Drawings were made from life.

opposite sides of the animal and across the midline so that both longitudinal nerve cords were severed. The contraction pattern is typical of ditaxic locomotion. Ditaxic locomotion can be elicited when three interdigitating cuts are made (Fig. 2b) but movement could not be evoked in all preparations. Conduction did not occur around more than three overlapping cuts.

An interesting situation occurs when a cut is made behind the brain and continued posteriorly along the midline for most of the animal's length (Fig. 2c). Stimulation of the anterior portion of this strip results in ditaxic locomotion at the anterior end of the animal. Therefore the information is conducted posteriorly before being conducted anteriorly to the brain.

Lesions anterior to the brain

When a cut is made from the antero-lateral margin to a point midway in front of the brain (Fig. 3a), animals prodded behind the brain move ditaxically. However, the flap of anterior margin produced by the cut, does not take part in the process. The uncut portion performs normally but on the lesioned side muscular extension only occurs behind the cut. Movement waves are propagated posteriorly from this point.

If an intact animal is prodded along the anterior margin it twists to the opposite side before moving away (Fig. 3b). A cut made between the brain and the anterior stimulus site results in a different kind of reaction (Fig. 3c). The animal no longer performs the twisting avoidance reaction but retracts and backs away.

DISCUSSION

The major finding of this study is the presence of an apparent sensory nerve-net in *Notoplana,* a turbellarian. This system resembles those classically designated as nerve-nets (Bullock, 1965) with isopolar diffuse conducting systems. A physiological organization of this type has not been demonstrated in this group of animals before and is of some importance with regard to current concepts about the evolution of nervous systems. It should be mentioned, however, that two other types of conducting systems could be invoked to explain the results obtained here and neither of these can be completely excluded as the responsible systems. Conduction around lesions could occur in either the muscle layers or the epithelium. Possible anatomical grounds for muscle-muscle conduction have been found in tight junctions between adjacent sarcoplasmic membranes (Chien and Koopowitz, 1972), which could act as electrical synapses. However, for a number of reasons, muscular propagation is probably not responsible. First, one might expect a wave of contraction or extrusion to accompany conduction. This is not the case. Secondly, localized or extensive contractions caused by either mechanical or electrical stimuli do not themselves lead to ditaxic locomotion. Thirdly, it is difficult to envisage how information propagated in the muscle layers could be transferred to the brain—even if it were to reach the region of that organ. Possible pressure in stretch receptors in the muscles could translate contraction into removal activity, but one would expect these to be scattered throughout the organism and activated close to the site of stimulation.

Neuroidal conduction might feasibly be involved in the observed results. Epithelial, or neuroid, conduction is well known in animals as diverse as coelenterates (Mackie, 1970) and larval amphibia (Roberts, 1969). However, the problem of transferring the information to the brain from the epithelium remains. Jha and Mackie (1967) have shown at the ultrastructural cell level how the ectoderm might connect to the nervous system in *Cordylophora,* a hydrozoan. But, as yet, epithelial-neuronal connections are not known in the Turbellaria. Perhaps the best evidence that epithelial conduction is not involved comes from the different behavioral responses obtained from comparison between stimulation anterior to or behind the brain. One would not expect anterior stimulation to evoke backing away, while posterior stimulation causes forward locomotion if the same epithelial system was involved. It is difficult to see how an epithelial conducting system could differentiate between the positional information in the sites of the two stimuli.

The simplest hypothesis to explain the present results would be by invoking a diffuse conducting neural network. However, if the nerves are responsible then one might question why ditaxic locomotion cannot be evoked by electrical stimulation. Other attempts have been made to demonstrate diffuse conduction in the large nerve plexus of the polyclad *Planocera* (Gruber and Ewer, 1962; Ewer, 1965). These authors found that conduction (initiated by electrical stimulation to the brain) only occurred along direct routes to the brain and concluded that a diffusely conducting nerve-net did not exist. Perhaps they did not have the correct stimulus for evoking activity in the diffuse conducting systems. A similar kind of finding is reported in the echinoderms, where mechanical stimulation of the radial nerve cord produces impulses but electrical stimuli are ineffective (Cobb and Laverack, 1966). Pentreath and Cobb (1972) suggested that electrical stimuli might not elicit a response in echinoderms if the axons are small and highly insulated. This might hold for the sensory nerves in flatworms as well.

At present one cannot determine which part of the nervous system might be responsible for conduction around the lesions. Besides the two submuscular plexuses, there is also the possibility of a fine subepithelial or epithelial nerve-net. There are a number of reports in the literature of such nerve-nets in the turbellarians. Lentz (1968) has described an epithelial net in fresh-water planarians, but these networks have not been convincingly demonstrated in polyclads, either at the light or electron microscope level. Even in the simpler orders of the class an epithelial net appears uncommon (Bullock and Horridge, 1965), if indeed it actually occurs. One wonders if perhaps overlapping terminal branches of sensory cells might have been misinterpreted as an epidermal network by some of the earlier workers.

The functional significance of the anatomical network is puzzling. If a nerve-net has indeed been demonstrated in this work, then it appears to be confined to only the sensory system. Cutting nerves leading to anterior motor regions leave that part flaccid and incapable of joining in locomotory behavior patterns. It is also clear, however, that the entire sensory system is not arranged as a physiological nerve-net; not even for single modalities such as mechanoreception. Avoidance responses from the anterior edge of the animal indicate that very specific pathways are involved. Recruitment of certain set pathways is obviously important when it is necessary for an animal to localize the site of a stimulus if it must avoid the stimulus source. This is not important for stimuli behind the brain as normal locomotion will take the animal away from the source of irritation. The presence of a back-up system such as the nonspecific anterior system which causes the animal to back away from the stimulus has obvious selective advantages. But even if these nonspecific systems are based on an anatomical nerve-net it is difficult to see why this should dictate the form of the comparatively massive plexiform nervous system which exists. It is probably more reasonable to assume that some other, as yet unknown, function is responsible for the selective advantage that maintains this anastomosing arrangement.

I would like to thank Dr. R. D. Campbell for help with the cinematography and Drs. J. Arditti, R. K. Josephson and D. Stokes for their comments on the manuscript.

SUMMARY

(1). The response to mechanical stimuli in the polyclad flatworm, *Notoplana acticola,* is the initiation of ditaxic locomotion. The response to electrical stimuli is local contraction.

(2). Animals will respond to mechanical stimuli with ditaxic movements even if a series of cuts are made so that the stimulus must be propagated around lesions as in a nerve-net.

(3). Only the sensory side of the system is organized as a diffusely conducted system; motor control involves direct connections to the brain.

(4). Sensory stimuli that convey information about the location of a stimulus on the body also require direct routes.

LITERATURE CITED

Best, J. B., and J. Noel, 1969. Complex synaptic configurations in planarian brain. *Science,* **164**: 1070–1071.

Bullock, T. H., 1965. Comparative aspects of superficial conduction in echinoids and asteroids. *Amer. Zool.,* **5**: 545–562.

Bullock, T. H., and G. A. Horridge, 1965. *Structure and Function in the Nervous System of Invertebrates,* Vol. I. W. H. Freeman and Co., San Francisco, 798 pp.

Chien, P., and H. Koopowitz, 1972. The ultrastructure of neuromuscular systems in *Notoplana acticola,* a free-living polyclad flatworm. *Z. Zellforsch.,* **133**: 277–288.

Cobb, J. L. S., and M. S. Laverack, 1966. The lantern of *Echinus esculentus* (L). I. Gross anatomy and physiology. *Proc. Roy. Soc., London Series B.,* **164**: 624–640.

Ewer, D. W., 1965. Networks and spontaneous activity in echinoderms and platyhelminthes. *Amer. Zool.,* **5**: 563–572.

Gruber, S. A., and D. W. Ewer, 1962. Observations on the myo-neural physiology of the polyclad flatworm *Planocera gilchristi. J. Exp. Biol.,* **39**: 459–477.

Hadenfeldt, D., 1928. Das Nervensystem von *Stylochoplana maculata* und *Notoplana atomata. Z. Wiss. Zool.,* **133**: 586–638.

Hadzi, J., 1963. *The Evolution of the Metazoa.* MacMillan Co., New York, 499 pp.

Halton, D. W., and J. B. Jennings, 1964. Demonstration of the nervous system in the monogenetic trematode *Diplozoon paradoxum* by the indoxyl acetate method for esterases. *Nature,* **202**: 510–511.

Horridge, G. A., 1968. *Interneurones. Their Origin, Action, Specificity, Growth, and Plasticity.* W. H. Freeman and Co., San Francisco, 436 pp.

Koopowitz, H., and D. W. Ewer, 1970. Observations on the myo-neural physiology of a polyclad flatworm: inhibitory systems. *J. Exp. Biol.,* **53**: 1–8.

Lentz, T. L., 1968. *Primitive Nervous Systems.* Yale University Press, New Haven, 148 pp.

Mackie, G. L., 1970. Neuroid conduction and the evolution of conducting tissues. *Quart. Rev. Biol.,* **45**: 319–332.

Moore, A. R., 1923. The function of the brain in locomotion of the polyclad worm, *Youngia aurantiaca. J. Gen. Physiol.,* **6**: 73–76.

Morita, M., and J. L. Best, 1966. Electron Microscopic studies on planaria. III. Some observations on the fine structure of planarian nervous tissue. *J. Exp. Zool.,* **161**: 391–412.

Olmsted, J. M. D., 1922. The role of the nervous system in the locomotion of certain marine polyclads. *J. Exp. Zool.,* **36**: 57–66.

Pentreath, V. W., and J. L. S. Cobb, 1972. Neurobiology of Echinodermata. *Biol. Rev.,* **47**: 363–392.

Roberts, A., 1969. Conducted impulses in the skin of young tadpoles. *Nature,* **222**: 1265–1266.

Turner, R. S., 1946. Observations on the central nervous system of *Leptoplana acticola. J. Comp. Neurol.,* **85**: 53–65.

Reference: *Biol. Bull.*, **145**: 360–372. (October, 1973)

THE EFFECT OF CYTOCHALASIN B UPON TAIL RESORPTION AND METAMORPHOSIS IN TEN SPECIES OF ASCIDIANS[1]

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Metamorphosis of the free swimming tadpole larva is a cardinal event in the life cycle of all ascidians, with the exception of a few anurous species in the families Molgulidae and Styelidae, which exhibit direct development (Berrill, 1931; Millar, 1971). There are many variations in the details of metamorphosis in different families. The following events are characteristic of most species.

Metamorphosis begins at the moment the larva settles. Settling is effected by the secretion of a sticky substance by the adhesive papillae and may involve rapid elongation or eversion of the papillae in some species. Settling is followed by resorption of the tail, retraction of the adhesive papillae, retraction or collapse of the sensory vesicle, emigration (in some species) of blood cells across the epidermis into the tunic, enlargement or elongation of epidermal ampullae, expansion of the tunic of the trunk, loss of the outer cuticular layer of tunic (comprising the fins of the tail), and a gradual rotation of the viscera and siphons through an arc of about 90 degrees. The axial complex of the tail is phagocytized and parts of the larval neurosensory system later undergo histolysis.

Postlarval development is highly variable. In some species the visceral organs are well differentiated in the larva and feeding begins within one to several hours (Berrill, 1935; Cloney, 1972). In other species, rudiments of the viscera are less well developed and must undergo differentiation over a period of several days before feeding begins (Grave, 1926, 1944; Cloney, 1961).

The morphogenetic movements associated with tail resorption have been studied extensively. In representative species of the families Polyclinidae, Clavelinidae, and Didemnidae (classification, Berrill, 1950), the caudal epidermis contracts during tail resorption and forces the axial complex (notochord, muscle and nerve cord) into the trunk (Cloney, 1963, 1972; Cloney and Lash, 1972). Contraction of the epidermis in *Amaroucium constellatum, Distaplia occidentalis* and *Diplosoma macdonaldi* is associated with the alignment of prominent arrays of cytoplasmic filaments in the epidermal cells (Cloney, 1966, 1972). In *Boltenia villosa* (family pyuridae) the notochordal cells appear to be contractile and the epidermis is evidently passive; changes in the organization of cytoplasmic filaments were found only in the notochordal cells (Cloney, 1969).

In the past few years, oriented arrays of cytoplasmic filaments (microfilaments) have been found in a wide variety of metazoan cells, which had been fixed while

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undergoing movements or changes of shape (*e.g.*, Baker and Schroeder, 1967; Schroeder, 1968, 1970; Arnold, 1969; Tilney and Marsland, 1969; Szollosi, 1970; Wessells, Spooner, Ash, Bradley, Luduena, Taylor, Wrenn, and Yamada, 1971; Crawford, Cloney and Cahn, 1972). The discovery that cytochalasin B (CCB) interferes with cytokinesis (Carter, 1967), and a variety of morphogenetic movements, and simultaneously interferes with the organization of microfilaments (Schroeder, 1969, 1970, 1972; Wessells *et al.*, 1971; Wrenn, 1971; Yamada, Spooner and Wessells, 1971; Spooner and Wessells, 1972) suggested a convenient method of analyzing morphogenetic movements in a variety of ascidians.

We have determined that CCB reversibly inhibits tail resorption and disrupts the organization of arrays of cytoplasmic filaments in the contractile epithelial cells (Lash, Cloney and Minor, 1970; Cloney, Lash and Minor, 1971; Cloney, 1972). In this paper we will describe the effect of CCB on tail resorption in ten species of ascidians including representatives of 8 different families and 3 suborders. We will show that there is a considerable variation in the effective concentration of the drug when it is used with different groups of ascidians, and that within a single species, discrete morphogenetic events, characteristic of metamorphosis, are differentially affected.

MATERIALS AND METHODS

Ten species of ascidians, representing the following suborders (Berrill, 1950), were used for the cytochalasin B experiments: Aplousobranchia; *Distaplia occidentalis, Diplosoma macdonaldi, Amaroucium constellatum.* Phlebobranchia; *Ciona intestinalis, Perophora viridis.* Stolidobranchia; *Botryllus schlosseri, Styela partita, Boltenia villosa, Molgula citrina, M. manhattensis.* Ultrastructural studies of the localization of cytoplasmic filaments were performed on *D. occidentalis, D. macdonaldi, A. constellatum, B. schlosseri, B. villosa, M. manhattensis,* the phlebobranch *Ascidia callosa,* and the stolidobranchs *Pyura haustor* and *Styela gibbsii* (methods of fixation have been described elsewhere; Cloney, 1964, 1966, 1972).

D. occidentalis, D. macdonaldi, B. villosa, P. haustor, S. gibbsii and *A. callosa* were obtained near the Friday Harbor Laboratories, University of Washington. All other species were obtained at the Marine Biological Laboratory, Woods Hole, Massachusetts. Tadpoles of oviparous species (*C. intestinalis, B. villosa, M. manhattensis, S. partita*) were used for experimentation 8–18 hours after hatching. Since the tadpoles from the ovoviviparous species (*D. occidentalis, D. macdonaldi, A. constellatum, P. viridis, B. schlosseri, M. citrina*) metamorphose shortly after they escape from the adult colonies, these species were treated with cytochalasin B within minutes or a few hours after the tadpoles were released.

For experimental observation, the tadpoles were placed in culture dishes containing filtered sea water (FSW), or FSW plus cytochalasin B. Periodic observations were made with either a dissecting microscope, a bright field or a Zeiss Nomarski differential interference microscope. Time lapse films of some species (*D. occidentalis, D. macdonaldi, B. villosa, A. constellatum, C. intestinalis, P. viridis, M. citrina, M. manhattensis*) were made with a Series 500 Sage cinephotomicrographic appartus, or a Kodak Reflex S, equipped with an L. W. photo intervalometer. Exposure intervals ranged from 0.5 to 8 frames per second.

Cytochalasin B was generously provided by S. B. Carter of Imperial Chemical

TABLE I

Effective concentration of cytochalasin B which arrests or prevents the onset of tail resorption (micrograms/ml of sea water)

	CCB dosage	Specimens observed
Order Enterogona		
Suborder Aplousobranchiata		
Distaplia occidentalis	0.25–0.5	800
Diplosoma macdonaldi	0.25–0.5	600
Amaroucium constellatum	0.5 –1.0	3000
Suborder Phlebobranchiata		
Ciona intestinalis	1.0 –2.0	2000
Perophera viridis	1.0 –2.0	70
Order Pleurogona		
Suborder Stolidobranchiata		
Botryllus schlosseri	5–7	1000
Boltenia villosa	5–10	200
Stylela partita	5–10	1000
Molqula citrina	10–15	100
Molgula manhattensis	15–20	2000

Industries Ltd., Macclesfield, Cheshire, England. A stock solution was prepared by dissolving 10 mg of CCB in 10 ml of dimethylsulfoxide (DMSO). Aliquots of 0.5 ml in glass tubes were kept at −10° C until use. A fresh stock solution was made every 10–14 days, since cytochalasin B absorbs to the glass container, and the effective dose level (by volume) increases with time. For experimental use, the stock solution was diluted with fresh FSW to concentrations ranging from 0.25 μg/ml to 50 μg/ml. Controls were treated with DMSO in FSW at the same concentration used in the cytochalasin B experiments.

RESULTS

Inhibition of metamorphosis

The intiation of metamorphosis can be completely inhibited by cytochalasin B, in all ten species of ascidians tested (Table I). It is clear that the effective concentration of the drug is distinctly different in the three suborders represented.

Arresting tail resorption

All ten species were tested to determine the effective concentration that would arrest tail resorption after the process had begun. The effective concentration was identical to that which prevented the initiation of tail resorption (Table I).

Reversal of the effects

Aplousobranchs. If the concentrations are minimal, and if the tadpoles are washed shortly after tail resorption is stopped, the effects of cytochalasin B are reversible. When CCB is added during tail resorption, the tail tends to push out again while in the presence of the drug. This is due to the relaxation of the

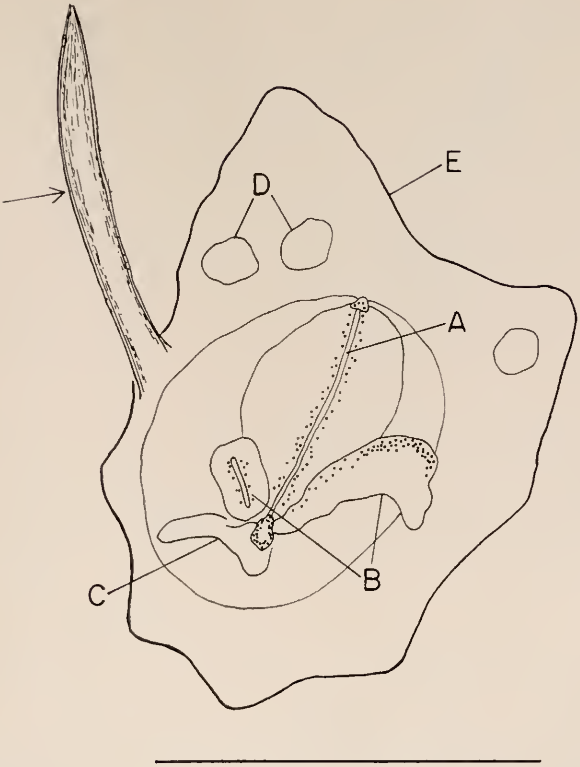

Figure 1. *Botryllus schlosseri.* Tadpole was kept in cytochalasin B (10 μg/ml) until signs of metamorphosis were observed (discharge of adhesive papillae, tunic swelling), then rinsed in fresh sea water. After seven days, the unresorbed tail (arrow) still protruded from the developing organism. Scale line is 1 mm. Abbreviations are: A, Endostyle of branchial basket; B, Degenerating (right) and take-over buds; C, Intestine; D, Ampullae; E, Tunic.

epithelial cells and uncoiling of the axial complex. When treated larvae are placed in fresh sea water, the tails begin to shorten again within 0.5 to 4 minutes, depending upon the duration of exposure to CCB (*A. constellatum, B. schlosseri, C. intestinalis, P. viridis*). In most instances complete recovery occurs only if the animals are washed within a few minutes after the tail stops shortening (*D. occidentalis* and *D. macdonaldi*), but many of the larvae of *A. constellatum* can recover and completely resorb their tails after periods as long as 35 minutes in the presence of CCB. Variations in the length of time in which recovery can be effected have been observed, and this may reflect differences in batches of tadpoles.

Tail resorption in *A. constellatum* was reversed four consecutive times within 20 minutes (four additions of CCB, four rinses). After the fourth reversal, the epidermis ruptured and the axial complex uncoiled. The epidermal cells separated from their neighbors, rounded up, and appeared as isolated beads on the surface of the axial complex.

Phlebobranchs. With the exception of a longer recovery time in the phlebo-

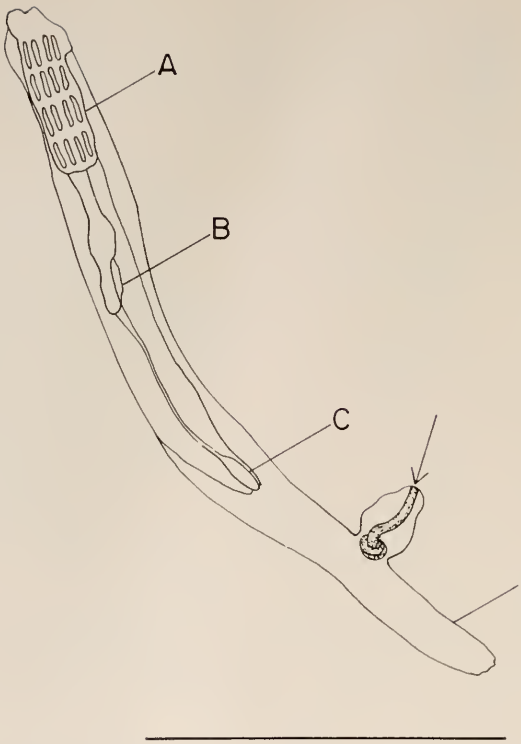

FIGURE 2. *Amaroucium constellatum.* Tadpole was treated with cytochalasin B (5 μg/ml), and rinsed before tail resorption was initiated. The tail underwent partial resorption (arrow). Scale line is 1 mm. Abbreviations are: A, Branchial basket; B, Intestine; C, Heart; D, Tunic.

branchs, the re-extension of the tail in the presence of CCB was similar to the events recorded in the aplousobranchs. Re-extension of the tail and recovery of movement sometimes took as long as 2–3 hours. Repetitive reversal was not tried with these species.

Stolidobranchs. In all five species examined, the stolidobranchs showed the following attributes: (1) They required the highest concentration of CCB to effectively inhibit tail resorption, (2) they required the longest time for CCB to stop tail resorption, and (3) they required the longest time for re-extension to occur. After adding CCB it took up to ten minutes for tail resorption to cease, and in some instances (*S. partita*) reinitiation of tail resorption after washing took as long as 4–5 hours. The completion of tail resorption sometimes took as long as 10 hours (*S. partita, M. manhattensis*).

Cytochalasin B, tail resorption, and metamorphosis

Reversal of the effects of cytochalasin B does not always result in normal tail resorption, but unless the concentration is high (2–3 times the minimum dose), general metamorphosis and postlarval development resumes. Frequently the organisms can continue development with an unresorbed, or partly resorbed tail protruding from the body wall (Fig. 1). These animals developed and behaved normally during the time of observation (8–10 days).

If tadpoles (*A. constellatum, B. schlosseri*) are placed in CCB, then rinsed before tail resorption is initiated, the process of tail resorption and metamorphosis is usually normal. In a few cases the tail did not undergo completely normal resorption. In *A. constellatum,* after completion of tail resorption, a compact, cone-shaped mass of contracted caudal epidermal cells persists near the base of the former tail (Fig. 2). These epidermal cells were often unable to contract further and invert the epithelial cone as occurs normally (Cloney, 1966). In *B. schlosseri,* if resorption is not completed, a small stump of tissue remains covered by a thin epithelium. In neither instance do these minor abnormalities have any effect upon further postlarval development.

Observations on normally metamorphosing tadpoles of *A. constellatum* have shown that tadpoles occasionally undergo metamorphosis without tail resorption. Excision of the tail of *A. constellatum, D. occidentalis, D. macdonaldi, B. villosa. P. viridis,* and *B. schlosseri* does not interfere with subsequent metamorphosis of the trunk. Thus inhibition of tail resorption with cytochalasin B would not necessarily be expected to affect the other characteristic events of metamorphosis.

Cytochalasin B and metamorphosis

In the aplousobranchs (*D. occidentalis, D. macdonaldi,* and *A. constellatum*), both the evanescent larval organs and the visceral organs of significance in post-larval life are well differentiated. Although *M. citrina* is ovoviviparous, and undergoes metamorphosis shortly after hatching, its structure is relatively simple (Grave, 1926). The other species tested in the stolidobranchs (*M. manhattensis, B. villosa, S. partita*), as well as *C. intestinalis*, are oviparous species. After external fertilization, and a relatively short (8–32 hours, 12°–22° C) period of development, a simple tadpole is hatched and may swim for several days before undergoing metamorphosis. *M. manhattensis, M. citrina, B. villosa, S. partita,* and *C. intestinalis* all undergo extensive differentiation after metamorphosis, before the adult organs become functional.

With these two distinctive types of development, from highly differentiated to simple larvae, it was possible to test the effects of cytochalasin B on processes other than tail resorption. As mentioned previously, the trunk can undergo normal metamorphosis even if the tail is only partly resorbed (*A. constellatum, D. occidentalis, B. schlosseri, D. macdonaldi, B. villosa*). At minimal dosage levels and continuous treatment, *A. constellatum* and *B. schlosseri* can develop with the tail protruding from the body wall. After several days the tail undergoes histolysis and falls off.

Tadpoles of *A. constellatum* and *B. schlosseri* were submitted to two types of treatment. In one, the tadpoles were continually exposed to cytochalasin B for periods ranging up to 7 days. The other treatment consisted of exposing animals to different concentrations of the drug, then rinsing them with FSW. In concentrations of 1.0 μg/ml of CCB, specimens of *A. constellatum* fail to resorb their tails, but otherwise develop normally.

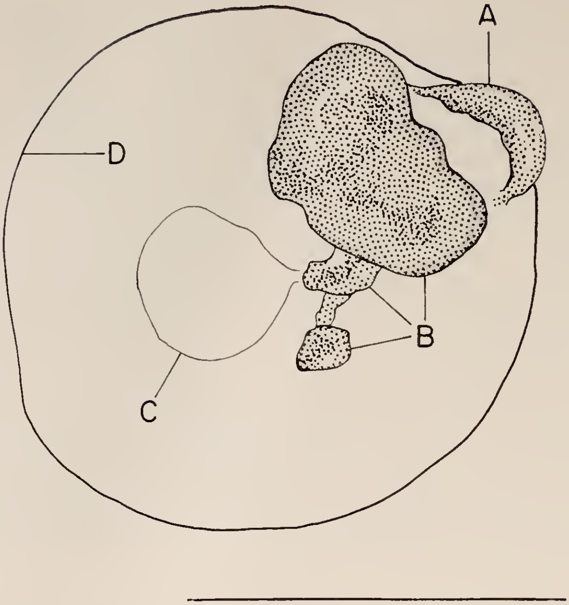

FIGURE 3. *Botryllus schlosseri.* Tadpole was kept continuously in cytochlasin B (10 μg/ml) for four days. The terata formed had a beating heart (A) and disorganized tissue (B). Scale line is 1 mm. Abbreviations are: A, Heart; B, Tissue mass; C, Vesicle; D, Tunic.

In control dishes containing only filtered sea water, up to five per cent of these tadpoles metamorphosed without resorbing their tails.

After a few hours, sometimes as long as 10 hours, in solutions of CCB, the caudal epidermal cells separate, and collect in clumps of cells along the length of the tail. The time at which this happens varies, and may be related to the developmental age of the tadpole. Tadpoles which metamorphose immediately after spawning may be older than those tadpoles which swim for longer periods before settling. All fully developed tadpoles are released each day in a brief period following exposure to light, whereas the development of the embryos is probably a continuous process. Thus with periodic release, the tadpoles would differ slightly in age.

In 5.0 μg/ml of CCB, the tail of *A. constellatum* is not resorbed, the tunic of the trunk however does begin to swell (a manifestation of metamorphosis), although there are few internal changes in the organism associated with metamorphosis, such as rotation of the visceral organs and opening of the siphons. Two-thirds of these animals show some inhibition of metamorphosis (30 tadpoles tested). The heart continues to beat for periods up to 4–5 days, but the animal remains small and malformed, and does not live past this time. At higher concentration, the animals die within a few days. Between 5–10 μg/ml of CCB there appears to be little, if any increase in size. It is probable that cytokinesis is strongly inhibited. At concentrations of 10 μg/ml, both tail resorption and general metamorphosis are completely inhibited (30 tadpoles tested).

With minor differences, the results of prolonged exposure of *B. schlosseri* to CCB are similar to those obtained with *A. constellatum.* The cytoplasmic filaments associated with cellular contraction are in the apical region of the epidermal cells of *A. constellatum* (Cloney, 1966), whereas they are in the basal part of the contracting epidermal cells of *B. schlosseri* (Minor and Lash, unpublished). At increasing concentrations (10 μg/ml), *B. schlosseri* acquired more abnormalities, and there is very little evidence of growth or morphogenesis. At the higher dosage levels, the organism transforms into a small (1.5 mm diameter) disorganized mass of tissue with a beating heart (Fig. 3). These terata continue "living" for a few days, then degenerate. This is further evidence that higher concentrations of cytochalasin B are required to inhibit growth and morphogenesis than is necessary to inhibit tail resorption.

Discussion

Configurational changes of a variety of cells have been correlated with the presence, in specific localized areas of the cytoplasm, of oriented arrays of 50–70 Å (diameter) filaments.

Organized arrays of filaments associated with the plasmalemma of the cleavage furrow have been implicated in the mechanism of cytokinesis in hydrozoans (Schroeder, 1968; Szollosi, 1970), polychaetes (Szollosi, 1970), cephalopods (Arnold, 1969), echinoids (Tilney and Marsland, 1969; Schroeder, 1969; 1972), and amphibians (Selman and Perry, 1970; Bluemink, 1970). They have also been implicated in the contraction of cells involved in the morphogenesis of the neural tube (Baker and Schroeder, 1967; Schroeder, 1970; Burnside, 1971), the salivary gland, pancreas, and oviduct (Spooner and Wessells, 1972; Wessells and Evans, 1968; Wrenn, 1971). In addition, these filaments appear to be involved in the movement of axonal growth cones (Yamada *et al.*, 1971), slime mold movements (Wohlforth-Bottermann, 1964), movements of glial cells (Spooner, Yamada and Wessells, 1971), platelet contraction (Shepro, Belamarich, Robblee and Chao, 1970), macrophage movement and endocytosis (Allison, Davies and de Detris, 1971), chemotaxis of polymorphonuclear leukocytes (Becker, Davis, Estensen and Quie, 1972), amoeboid movement (Nachmias, 1968), and the movement of human lymphocytes (Smith, Ridler and Faunch, 1967). It has even been proposed by Jones (1966) that cell reaggregation (specifically, sponge cells) rely upon a mechanism involving cytoplasmic contractile elements (*viz.* cytoplasmic filaments).

For all species of ascidians thus far analyzed (*D. occidentalis, D. macdonaldi, A. constellatum, B. villosa, B. schlosseri*), the evidence strongly suggests that the cytoplasmic filaments provide, in an as yet unknown manner, the motive force necessary for tail resorption (Cloney, 1966; 1969; 1972). Projecting from data obtained from the variety of systems previously mentioned, it was predicted that cytochalasin B would interfere with tail resorption. In all ten species tested, representing three different suborders and eight different families, cytochalasin B prevented the initiation of tail resorption. If treated with the drug after tail resorption had begun, shortening of the tail was arrested. In the aplousobranchs, phlebobranchs, and the stolidobranchs (with the exception of *B. villosa*), the contractile tissues then relaxed and the tail began to push out again. In *A. constellatum* this process of reversal could be repeated four times.

Ultrastructural studies of *A. constellatum* and *D. occidentalis* have shown that when specimens are fixed immediately after tail resorption is inhibited with CCB, most of the central and subterminal arrays of filaments which normally span the apex of the contracting epidermal cells are disorganized. The only remaining organized arrays of filaments are short tufts or patches attached to the plasmalemma (Lash, Cloney and Minor, 1970; Cloney, 1972). The presence of the terminal tufts of filaments suggests that treatment with CCB does not completely degrade the filaments into subunits. It is more likely that the drug disrupts the binding force between overlapping unattached filaments.

Although it has been reported that cytochalasin B has a pronounced inhibitory effect upon polysaccharide synthesis (Sanger and Holzter, 1972), data from others indicate that this observed inhibition is an artifact, and that it is the result of cytochalasin B rapidly inhibiting the transport of such molecules as glucose, glucosamine, D-2-deoxyglucose (Estensen and Plagemann, 1972; Kletzien, Perdue and Springer, 1972; Zigmond and Hirsch, 1972) and nucleosides (Plagemann and Estensen, 1972). The effect of cytochalasin B on ascidians reported in this paper occurs so rapidly that one would not expect general synthetic processes to be involved in the mechanism of inhibition. It is not however, possible to determine whether CCB acts directly or indirectly upon the filaments.

The effective range of concentration of cytochalasin B which prevents tail resorption exhibited a striking phylogenetic correlation. Tail resorption in all three species of aplousobranchs (*D. occidentalis, D. macdonaldi, A. constellatum*) was effectively blocked at a concentration range of 0.25–1 μg/ml. In the phlebobranchs (*C. intestinalis* and *P. viridis*) the effective concentration range was 1–2 μg/ml. In the stolidobranchs (*B. schlosseri, B. villosa, S. partita, M. citrina* and *M. manhattensis*), resorption was blocked in a concentration range from 5–20 μg/ml. The two molgulids tested (representing the most phylogenetically advanced family of this suborder, Berrill (1950)) required the highest concentration (*M. citrina*, 10–15 μg/ml; *M. manhattensis*, 15–20 μg/ml). It is not possible to state whether the subordinal relationship to effective concentration will hold true when more species are tested.

There are several possible explanations for the differences in effective concentrations found. In the aplousobranchs, which are affected by the *lowest* concentrations, the most superficial tissue, the caudal epidermis, is contractile and the organized arrays of filaments are localized in the most superficial region (apical cytoplasm) of these cells (Cloney, 1966). In contrast, in the stolidobranchs, which require a 5–10 fold higher concentration, the organized arrays of filaments are located in the basal region of the epidermal cells (*B. schlosseri*), or in the notochordal cells (*B. villosa*). Thus there is a correlation between the effective CCB concentration and the locus of the contractile organelles. The localization of cytoplasmic filaments in 13 species of ascidians is summarized in Table II. In *D. occidentalis, D. macdonaldi, A. constellatum, C. intestinalis, A. callosa, B. schlosseri, B. villosa, M. manhattensis, S. gibbsii* and *P. haustor* the localization of filaments (as indicated by the symbol +) was determined by electron microscopy (Cloney, 1966, 1969, 1972; Lash, Cloney and Minor, 1970; Wessells, Spooner, Ash, Bradley, Ludena, Taylor, Wrenn and Yamada, 1971; Cloney, unpublished; Minor and Lash, unpublished). The localization of filaments in *P. viridis* was deduced by

TABLE II

Localization of major arrays of cytoplasmic filaments in thirteen species of ascidians undergoing tail resorption

	Caudal epidermis (apical)	Caudal epidermis (basal)	Notochord
Order Enterogona			
Suborder Aplousobranchiata			
Distaplia occidentalis	+		
Diplosoma macdonaldi	+		
Amaroucium constellatum	+		
Suborder Phlebobranchiata			
Ciona intestinalis	+		
Perophora viridis	+		
Ascidia callosa	+		
Order Pleurogona			
Suborder Stolidobranchiata			
Botryllus schlosseri		+	
Boltenia villosa			+
Molguia citrina			P
Molqula manhattensis			+
Pyura haustor			+
Styela gibbsii			+
Styela partita			P

analysis for birefringence (Lash and Reigart, 1965). For the other two species listed in the table, the probable position of the cytoplasmic filaments (as indicated with the symbol P) was deduced from time-lapse cinemicrographic observations, and changes in cell shape during tail resorption. Although the epidermis and notochord, in different species, appear to contain the major arrays of cytoplasmic filaments, the localization of filaments in these principal sites does not rule out the possibility of their occurrence in additional locations.

The correlation between effective CCB concentration and the locus of the contractile organelles suggests that the drug must be used in higher concentrations when the site of action is deeper within the organism. This would appear more reasonable if the data on *B. schlosseri* were not known. The filaments in the epidermal cells of *B. schlosseri* are only a few microns deeper within the cytoplasm of the epidermal cells than they are in epidermal cells of the aplousobranchs tested. Furthermore the contractile ampullae of *B. schlosseri* are composed of epidermal cells which have filaments in the basal cytoplasm (DeSanto and Dudley, 1969), and are 5–10 times more sensitive to the drug than the caudal epidermis.

An alternate explanation for the results is that the permeability of ascidian tissues to CCB varies along phylogenetic lines as well as among tissues of the same organism. Recent experiments by Bluemink and Luchtel (personal communication) on *Xenopus* eggs indicate that uncleaved eggs are impermeable to CCB. Localized inhibitory effects of the drug on cleavage are manifested when CCB is injected into egg cytoplasm.

Since nothing is yet known of the molecular aspects of the cytoplasmic filaments, or how their alignment is translated into motive force, it is not possible with assurance to say whether there may be different mechanistic principles involved in the different tissues. Recent work has indicated that 50–70 Å cytoplasmic filaments in many different cell types are actin, or very similar to actin (Jones, 1966; Ishikawa, Bischoff and Holtzer, 1969; Jones and Kemp, 1970; Tilney and Mooseker, 1971; Pollard, 1972). The cytoplasmic filaments in the contractile epidermal cells of *Distaplia occidentalis* have also been shown to bind heavy meromyosin and form "decorated filaments" like those that have been described in other non-muscular cells (Cloney, unpublished).

It appears that wherever contractile processes are associated with cytoplasmic filaments, these processes can be inhibited or prevented by treatment with cytochalasin B. Whatever the multiple effects of CCB may be, its disorganizing effect on arrays of actin-like filaments in non-muscular cells seems to be wide spread.

Tail resorption is just one event in ascidian metamorphosis. With higher concentrations of CCB, other metamorphic events are affected. Dosage levels which will affect other specific cellular processes have not been worked out in detail, but in all instances the minimum concentration inhibiting tail resorption is compatible with subsequent development in the aplousobranchs and phlebobranchs. In the stolidobranchs the effective concentration is much higher (5–20 μg/ml), and these higher concentrations result in developmental impairments. In some extreme instances, terata are formed. These terata are composed of a disorganized tissue mass and a pulsating heart. In general, contraction of striated muscle was not inhibited, but the results were variable. *A. constellatum* tadpoles swim for days in cytochalasin B, whereas caudal muscular activity is inhibited in *D. occidentalis* after 6 hours (Cloney, 1972). Although evidence is mounting that cytochalasin B may have multiple effects upon cellular processes, one general process which is inhibited is a contraction associated with the presence of cytoplasmic filaments in non-muscular cells.

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SUMMARY

Cytochalasin B reversibly inhibits tail resorption and disrupts the organization of arrays of cytoplasmic filaments in the contractile cells. The effects of cytochalasin B on tail resorption and metamorphosis has been examined in ten species of ascidians, including representatives of 8 different families and 3 suborders. There is a considerable variation in the effective concentration of the drug when it is used with different groups of ascidians, and within a single species, discrete morphogenetic events characteristic of metamorphosis are differentially affected. The effective range of concentration of cytochalasin B which prevents tail resorption exhibited a striking phylogenetic correlation.

LITERATURE CITED

Allison, A. C., P. Davies and S. de Detris, 1971. Role of contractile microfilaments in macrophage movement and endocytosis. *Nature New Biology*, **232**: 153–155.

Arnold, J. M., 1969. Cleavage furrow formation in a telolecithal egg (*Loligo pealli*). *J. Cell Biol.*, **41**: 894–904.

Baker, P. C., and T. E. Schroeder, 1967. Cytoplasmic filaments and morphogenetic movement in the amphibian neural tube. *Develop. Biol.*, **15**: 432–450.

Becker, E. L., A. T. Davis, R. D. Estesen and P. G. Quie, 1972. Cytochalasin B. IV. Inhibition and stimulation of chemotaxis of rabbit and human polymorphonuclear leukocytes. *J. Immunol.*, **108**: 396–402.

Berrill, N. J., 1931. Studies in tunicate development. II. Abbreviation of development in the Molgulidae. *Phil. Trans. Roy. Soc. London,* **219B**: 282–346.

Berrill, N. J., 1935. Studies on tunicate development. III. Differential retardation and acceleration. *Phil. Trans. Roy. Soc. London,* **225B**: 225–326.

Berrill, N. J., 1950. *The Tunicata.* The Ray Society, London, 354 pp.

Bluemink, J. G., 1970. The first cleavage of the amphibian egg. *J. Ultrastruct. Res.*, **32**: 142–166.

Burnside, M. B., 1971. Microtubules and microfilaments in newt neurulation. *Develop. Biol.*, **26**: 416–441.

Carter, S. B., 1967. Effects of cytochalasin on mammalian cells. *Nature,* **213**: 261–264.

Cloney, R. A., 1961. Observations on the mechanism of tail resorption in ascidians. *Amer. Zool.*, **1**: 67-87.

Cloney, R. A., 1963. The significance of the caudal epidermis in ascidian metamorphosis. *Biol. Bull.*, **124**: 214–253.

Cloney, R. A., 1964. Development of the ascidian notochord. *Acta Embryol. Morphol. Exp.*, **7**: 111–130.

Cloney, R. A., 1966. Cytoplasmic filaments and cell movements: Epidermal cells during ascidian metamorphosis. *J. Ultrastruct. Res.*, **14**: 300–328.

Cloney, R. A., 1969. Cyotplasmic filaments and morphogenesis: The role of the notochord in ascidian metamorphosis. *Z. Zellforsch.*, **100**: 31–53.

Cloney, R. A., 1972. Cytoplasmic filaments and morphogenesis: Effects of cytochalasin B on ascidan tail resorption. *Z. Zellforsch.*, **132**: 167–192.

Cloney, R. A., J. W. Lash and R. R. Minor, 1971. Ascidian metamorphosis: Contractile tissues, cytochalasin B and hydrostatic pressure. *Biol. Bull.*, **141**: 382.

Cloney, R. A., and J. Lash, 1972. Ascidian metamorphosis: The role of contractile epidermis in tail resorption. [Sound film available from Education Development Center, 39 Chapel St., Newton, Massachusetts 02160.]

Crawford, B., R. A. Cloney and R. D. Cahn, 1972. Cloned pigmented retinal cells: The effects of cytochalasin B on ultrastructure and behavior. *Z. Zellforsch.*, **130**: 135–151.

De Santo, R. S., and P. L. Dudley, 1969. Ultramicroscopic filaments in the ascidian *Botryllus schlosseri (Pallas)* and their possible role in ampullar contractions. *J. Ultrasctruct. Res.*, **28**: 259–274.

Estensen, R. D., and P. G. W. Plagemann, 1972. Inhibition of glucose and glucosamine transport. *Proc. Nat. Acad. Sci.*, **60**: 1430–1434.

Grave, C., 1926. *Molgula citrina (Alder and Hancock).* Activities and structure of the free-swimming larva. *J. Morphol. Physiol.*, **42**: 453–467.

Grave, C., 1944. The larva of *Styela (Cynthia) partita:* Structure, function and duration of life. *J. Morphol.*, **75**: 173–191.

Ishikawa, H., R. Bischoff and H. Holtzer, 1969. Formation of arrowhead complexes with heavy meromyosin in a variety of cell types. *J. Cell Biol.*, **43**: 312–328.

Jones, B. M., 1966. A unifying hypothesis of cell adhesion. *Nature,* **212**: 362–365.

Jones, B. M., and R. B. Kemp, 1970. Inhibition of cell aggregation by antibodies directed against actomyosin. *Nature,* **226**: 261–262.

Kletzien, R. F., J. F. Perdue and A. Springer, 1972. Cytochalasin A and B: Inhibition of sugar uptake in cultured cells. *J. Biol. Chem.*, **247**: 2964–2966.

Lash, J., and J. R. Reigart, 1965. Studies on ascidian metamorphosis: Birefringence in tail epidermis. *Biol. Bull.*, **129**: 395.

Lash, J., R. A. Cloney and R. R. Minor, 1970. Tail resorption in ascidians: Effects of cytochalasin B. *Biol. Bull.*, **139**: 427–428.

Millar, R. H., 1971. The biology of ascidians. *Advan. Mar. Biol.*, **9**: 1–100.

Nachmias, V. T., 1968. Further electron microscope studies on fibrillar organization of the ground cytoplasm of *Chaos chaos. J. Cell Biol.,* **38**: 40–50.

PLAGEMANN, P. G. W., AND R. D. ESTENSEN, 1972. Cytochalasin B. VI. Competitive inhibition of nucleoside transport by cultured Novikoff rat hepatoma cells. *J. Cell Biol.*, **55**: 179–185.

POLLARD, T. D., 1972. Progress in understanding amoeboid movement at the molecular level. In press in K. Jeon, Ed., *Biology of Amoeba*. Academic Press, New York.

SANGER, J. W., AND H. HOLTZER, 1972. Cytochalasin B.: Effects on cell morphology, cell adhesion, and mucopolysaccharide synthesis. *Proc. Nat. Acad. Sci.*, **69**: 253–257.

SCHROEDER, T. E., 1968. Cytokinesis: Filaments in the cleavage furrow. *Exp. Cell Res.*, **53**: 272–318.

SCHROEDER, T. E., 1969. The role of "contractile ring" filaments in dividing *Arbacia* eggs. *Biol. Bull.*, **137**: 413–414.

SCHROEDER, T. E., 1970. The contractile ring. I. Fine structure of dividing mammalian (HeLa) cells and the effects of cytochalasin B. *Z. Zellforsch.*, **109**: 431–449.

SCHROEDER, T. E., 1972. The contractile ring. II. Determining its brief existence, volumetric changes, and vital role in cleaving *Arbacia* eggs. *J. Cell Biol.*, **53**: 419–434.

SELMAN, G. G., AND M. M. PERRY, 1970. Ultrastructural changes in the surface layers of the newt's egg in relation to the mechanism of its cleavage. *J. Cell Sci.*, **6**: 207.

SHEPRO, D., F. A. BELAMARICH, L. ROBBLEE AND F. C. CHAO, 1970. Antimotility effect of cytochalasin B observed in mammalian clot retraction. *J. Cell Biol.*, **47**: 544.

SMITH, G. F., M. A. C. RIDLER AND J. A. FAUNCH, 1967. Action of cytochalasin B on cultured human lymphocytes. *Nature*, **216**: 5120.

SPOONER, B., AND N. WESSELLS, 1972. An analysis of salivary gland morphogenesis: Role of cytoplasmic filaments and microtubules. *Develop. Biol.*, **27**: 38–54.

SPOONER, B. S., K. M. YAMADA AND N. K. WESSELLS, 1971. Microfilaments and cell locomotion. *J. Cell Biol.*, **49**: 595–613.

SZOLLOSI, D., 1970. Cortical cytoplasmic filaments of cleaving eggs: A structural element corresponding to the contractile ring. *J. Cell Biol.*, **44**: 129–209.

TILNEY, L. G., AND D. A. MARSLAND, 1969. A fine structural analysis of cleavage induction and furrowing in the eggs of *Arbacia punctulata*. *J. Cell Biol.*, **42**: 170.

TILNEY, L. G., AND M. MOOSEKER, 1971. Actin in the brush-border of epithelial cells of the chicken intestine. *Proc. Nat. Acad. Sci.*, **68**: 2611–2615.

WESSELLS, N. K., AND J. EVANS, 1968. Ultrastructural studies of early morphogenesis and cytodifferentiation in the embryonic mammalian pancreas. *Develop. Biol.*, **17**: 413–446.

WESSELLS, N. K., B. S. SPOONER, J. F. ASH, M. M. O. BRADLEY, M. A. LUDUENA, E. L. TAYLOR, J. T. WRENN AND K. M. YAMADA, 1971. Microfilaments in cellular and developmental processes. *Science*, **171**: 135–143.

WOHLFORTH-BOTTERMANN, K. E., 1964. Cell structures and their significance for amoeboid movement. *Int. Rev. Cytol.*, **16**: 61–131.

WRENN, J. T., 1971. An anaysis of tubular gland morphogenesis in chick oviduct. *Develop. Biol.*, **26**: 400–415.

YAMADA, K. M., B. S. SPOONER AND N. K. WESSELLS, 1971. Ultrastructure and function of growth cones and axons of cultured nerve cells. *J. Cell Biol.*, **49**: 614–635.

ZIGMOND, S. H., AND J. G. HIRSCH, 1972. Cytochalasin B: Inhibition of D-2-deoxyglucose transport into leukocytes and fibroblasts. *Science*, **176**: 1432–1434.

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OBSERVATIONS ON THE MARINE LEECH *CALLIOBDELLA CAROLINENSIS* (HIRUDINEA: PISCICOLIDAE), EPIZOOTIC ON THE ATLANTIC MENHADEN[1]

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Apart from the biological accounts of three European leeches, *Crangonobdella murmanica* by Selensky (1923), *Hemibdella soleae* by Llewellyn (1965) and *Oceanobdella blennii* by Gibson and Tong (1969) and Sawyer (1970), almost nothing is known about the biology of marine leeches, particularly from North America. This lack of information is partially due to their obscure taxonomy and partially due to the difficulty of working with these elusive parasites. Starting in the winter of 1970–71 an epidemic of a new piscicolid, *Calliobdella carolinensis,* on the Atlantic menhaden, *Brevoortia tyrannus,* gave us an unusual opportunity to investigate the behavior and population fluctuations of this marine leech (Sawyer and Chamberlain, 1972). Presented below are the results of three years of observations on various aspects of the biology of *C. carolinensis* in the vicinity of Charleston, South Carolina. The study constitutes the first account of a marine leech epizootic outbreak (epidemic).

METHODS

The majority of the observations was confined to two localities: (1) Beresford Creek, the type locality of *C. carolinensis:* and (2) the south end of Folly Beach. Both localities were chosen because of the accessibility and abundance of the leeches, as well as the prominent ecological differences between them.

Beresford Creek (32°53.2′N; 79°52.7′W) is a short estuarine tributary of the Wando River, Charleston County, South Carolina. The salinity is normally about 12–16‰. Like most estuarine streams in coastal South Carolina, the bottom and shoreline consist mainly of mud, mixed with occasional clumps of oysters. The water itself is heavily silted and contains many planktonic organisms which constitute food for the schools of juvenile menhaden which abound in the creek. The leeches were collected by periodically netting its host, the Atlantic menhaden, in a $20' \times \frac{1}{2}''$ square mesh otter trawl towed for approximately 2 hours. To determine infestation rates, the menhaden were immediately isolated into plastic bags for later laboratory examination. The unattached leeches found in the bottom of the boat and on shells, were collected for behavioral studies. Large samples of other species of fish collected along with the menhaden were also examined for leeches. The otter trawl technique was most successful in the winter months, presumably because the normally fast-swimming menhaden were too lethargic to escape the net.

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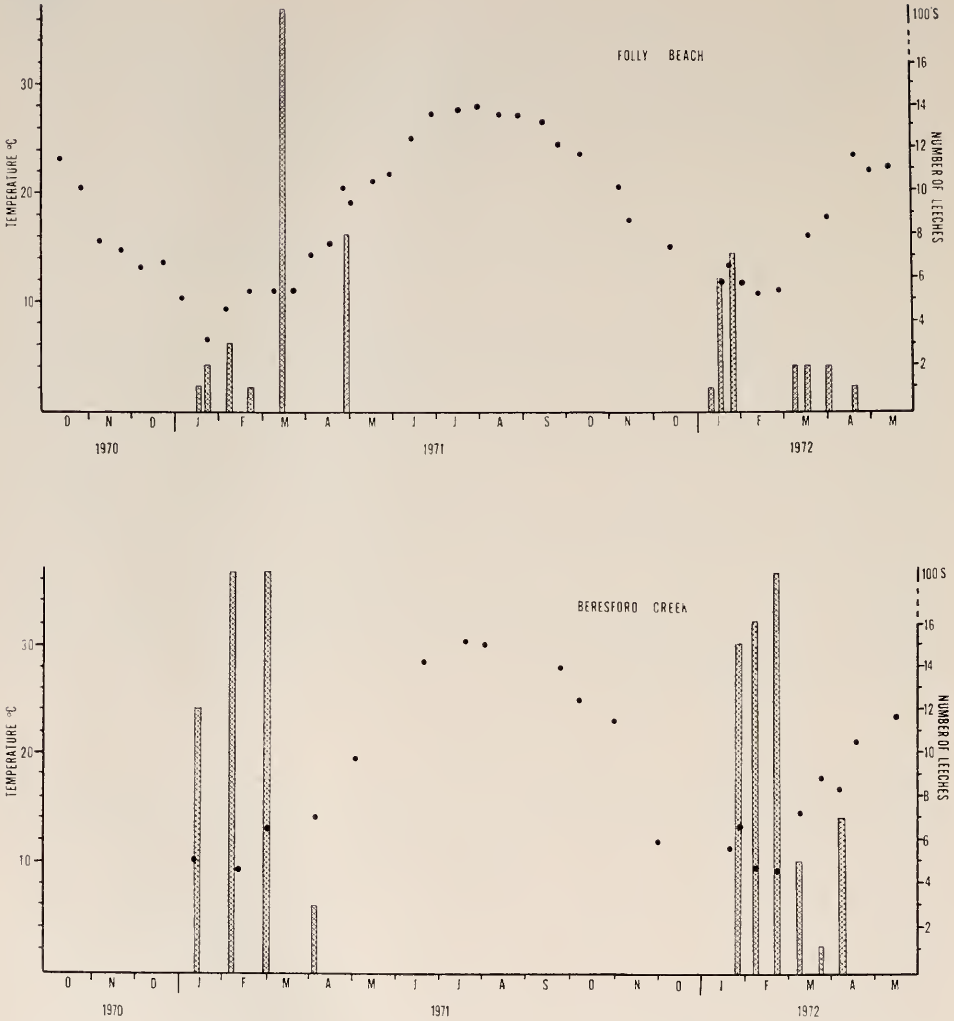

FIGURE 1. Temperature and seasonal occurrence of *Calliobdella carolinensis* at Folly Beach (upper graph) and at Beresford Creek (lower graph) from October 1970 to May 1972. The solid dots indicate the temperature of the water (°C) when each sample was taken. The vertical bars indicate the number of leeches collected on seaweeds at Folly Beach and on menhaden at Beresford Creek.

The south end of Folly Beach (32°38.3′N; 79°58.5′W) constitutes the north bank of the mouth of the estuarine Folly River. The salinity was normally about 28–32‰, but varied from 25.8–35.0‰. The beach area is usually silty and turbulent. In a one hour period the leeches were hand collected on the green seaweeds (primarily *Ulva lactuca*) free-floating in the open surf. During the winter months a few leeches were collected on menhaden caught in a 65′ × 6′ × ¼″ square mesh bag seine dragged in the surf as a part of a separate study on the surf zone fish of Folly Beach.

In addition to the above observations, our understanding of the distribution of this leech is augmented by random collections at various localities along the North and South Carolina coasts either by capturing menhaden in nets or by sampling free-floating green seaweeds. Several attempts to collect the leeches with plankton nets (1 m diameter, 0.5 mm square mesh) were also successful in certain tidal creeks near Charleston.

In the laboratory the leeches were placed into aquaria (salinity 28‰) of various sizes in a refrigerator and maintained in constant dark at 9° C. The leeches, which were not fed, thrived for months under these conditions.

RESULTS

Population dynamics and distribution

In spite of extensive research on the parasites of the Altantic menhaden, *C. carolinensis* was practically unknown until the population explosion of January–March 1971 in which hundreds, if not thousands, of leeches were found with almost every collection of menhaden. Local commercial fisherman verified that during this period the leeches accumulated on the decks of their boats in great numbers and were often found on the hands and feet of the fishermen. Prior to this population explosion the only records of *C. carolinensis* in South Carolina were three individuals collected unattached to a host in Charleston Harbor near Fort Johnson on 14 March 1963, and one individual collected from a menhaden at Dewees Inlet, Isle of Palms, South Carolina on 9 February 1970. The infestations of January–March 1972 and 1973, while still high, had declined noticeably from the same period in 1971.

C. carolinensis displays a remarkable seasonal occurrence which correlates with the temperature of the sea water (Fig. 1). For example, at Beresford Creek during the two full winters of the study, the first individuals of *C. carolinensis* were encountered on menhaden on 12 January 1971 and 26 January 1972 when the water temperature had dropped to 10.1° C and 13.3° C, respectively. On 13–14 December 1972 (water 15.0°, 16.8° C, respectively) small individuals were found on the Blueback herring, *Alosa aestivalis* (see discussion). At Folly Beach the first individuals were encountered, unattached to a host, on 12 January 1971 and 9 January 1972, when the water temperature had dropped to 9.5° C and 14.9° C, respectively.

The presence of the first leeches also strongly correlates with the presence of menhaden in the various collecting nets. At Beresford Creek the first menhaden were collected in the otter trawl on the same dates mentioned above. At Folly Beach the first menhaden were collected on 21 January 1971 and 23 January 1972, when the water temperature was 6.4° C and 13.2° C, respectively. It is known that menhaden occur in these regions throughout the year, but are caught in the nets in great number only from January to early March, presumably because the fish become lethargic in the cold water (see discussion). This lethargy of the menhaden probably plays an important role in the life cycle of *C. carolinensis*.

Leeches reached their greatest abundance when the water was near its coldest temperature. For example, at Beresford Creek during the two full winters of the study the greatest number of individuals were encountered on menhaden on 2 March 1971 and 23 February 1972, when the water was 13.1° C and 9.4° C, respectively. At Folly Beach the greatest number of individuals were encountered on seaweeds on 13 March 1971 and 29 January 1972, when the water temperature was 10.8° C and 11.7° C, respectively.

With the advent of rapidly warming water, the leeches became scarcer and eventually disappeared by the end of April. For example, at Beresford Creek the last individuals were encountered on menhaden on 5 April 1971 and 7 April 1972 when the water was 14.4° C and 17.0° C, respectively. At Folly Beach the last individuals were encountered on seaweeds 29 April 1971 and 19 April 1972, when the water was 17.0° C and 23.2° C, respectively. An attempt on 1 June 1972 to collect menhaden from a commercial shrimp trawler in the open sea three miles off Folly Beach yielded 44 large (137–207 mm, SL) uninfected menhaden. A similar attempt on 29 October yielded 67 uninfected menhaden (128–197 mm, SL). Collections of menhaden throughout the summer and fall of 1972 failed to turn up any leeches. In fact in 1970, 1971 and 1972 no leeches were encountered from May until the following mid-December or early January in spite of repeated attempts to collect them on the seaweeds or menhaden. However, relatively few menhaden were collected in the estuarine creeks during this period possibly because of the avoidance of the otter trawl by the fast-swimming menhaden (Wilkens and Lewis, 1971). Menhaden were collected during this time by local fishermen using other techniques.

The population structures of both the Beresford Creek and the Folly Beach populations of *C. carolinensis* were examined by measuring the total lengths of relaxed individuals with the aid of an ocular micrometer. At Beresford Creek 210 individuals, ranging in length from 3.0 to 29.0 mm (mode, 9.0 mm), were collected on 23 February 1972 from the mouth cavities of 41 menhaden. At Folly Beach 91 unattached individuals, ranging in length from 5.0 to 25.0 mm (modes, 11.0 and 17.5 mm) were collected on 13 March 1971 from a large clump of floating *Ulva*.

The Beresford Creek population, taken directly from the host, contained more immature (3 to 9 mm) individuals than the unattached Folly Beach population. This is consistent with the normal behavior of the Piscicolidae, *i.e.*, the mature individuals are the first to leave the host to breed. Becker and Katz (1965a) made similar observations on *Piscicola salmositica*.

In South Carolina *C. carolinensis* is restricted to the mouths and inlets of estuarine rivers and streams from North Inlet, Georgetown, south to Hunting Island State Park below Beaufort. It has not yet been found south of South Carolina, nor does it occur along the lengthy, sandy beaches of the Grand Strand area of South Carolina north of Georgetown to Cherry Grove. This is presumably due to the absence of large estuaries. In spite of extensive menhaden research in North Carolina *C. carolinensis* has not yet been reported from that state. However, several individuals were recently recovered in Virginia from the collections of the Virginia Institute of Marine Science (VIMS). Three individuals were collected on 25 March 1959 from menhaden in the York River. Another individual was found on 21 January 1965 unattached to a host in a suspended tray of oysters at the VIMS pier, York River, Virginia (37°14′46″N ; 76°30′02″W). In spite of extensive parasitological research by VIMS, it has not been found there since.

Like most truly estuarine invertebrates, *C. carolinensis* displays a remarkable tolerance to fresh water. During the study it was collected in water ranging from 32.5‰ at 10.5° C to 4.5‰ at 19.4° C, but most individuals were collected in water from 10 to 20‰. In the laboratory a series of salinity tolerance experiments was undertaken in which leeches were taken from water at 28‰ and placed immediately into salinities of 0, 1.62, 3.24, 6.48, 12.19 and 25.85‰ (Sawyer, in preparation). Each container held five adult leeches, and the experiment was undertaken at 10° C as well as at 21.5° C. At each salinity the leeches clearly had a greater tolerance to fresh water at the lower temperature than at the higher temperature. For example, they withstood pond water 12 days at 21.5° C and for 51 days at 10° C. At 10° C they lived indefinitely from 3.24 to 25.85‰, while at 21.5° C they lived indefinitely only in 25.85‰. When the leeches were slowly conditioned to pond water the results were similar.

Feeding

The infestation rate of *C. carolinensis* on menhaden in South Carolina estuaries is remarkably high. For example, at Beresford Creek on 23 February 1972, 67.7% of the 41 menhaden (85 to 138 mm, SL) collected were infested with 210 leeches. Most of the fish harbored from 2 to 17 leeches, but two fish (115 and 126 mm, SL) each had 36 leeches in their mouth cavities. On the whole it appears that the larger fish harbor more parasites. On 19 December 1972, 348 leeches (mostly from 4 to 10 mm in length) were found in the mouth cavity of one large menhaden (227 mm, SL). Similarly, on 8 February 1973 of 11 menhaden examined 10 (99–153 mm, SL) had from 4 to 75 leeches in their mouth cavities. Other samples of menhaden yielded similar results, some of the smaller samples occasionally yielding up to 100% infestation. Leeches were most commonly found on menhaden of 96 to 126 mm, SL, but some were found on fish of 91 mm to 277 mm, SL.

Throughout the three years of the study all sizes of *C. carolinensis* collected from the menhaden occurred exclusively in their mouth cavities, none being found on the body nor the fins. None of the leeches was actually seen sucking the blood of the fish, but the crops of most were engorged with blood. In the laboratory, however, the leeches were observed feeding on the clingfish, *Gobiesox strumosus*, the white catfish, *Ictalurus catus*, and the mullet *Mugil cephalus*. In each case feeding was on various regions of the body outside the mouth cavity and lasted for at least 6 to 8 hours. The body of the leech was sharply bent and the suckers were closely positioned. In some cases the fish reacted with a frenzied darting motion which occasionally dislodged the leeches.

Under natural conditions *C. carolinensis* feeds almost exclusively on clupeid fish, particularly on menhaden. In fact, the overwhelming majority of the many hundreds of leeches collected from fish in the Charleston area were taken from the Atlantic menhaden, *Brevoortia tyrannus*. The most notable exception was the occurrence on 21 April 1971 of numerous leeches in the mouth cavities of the Blueback herring, *Alosa aestivalis* (Mitchill), another clupeid fish with feeding habits similar to those of *B. tyrannus*. On 13–14 December 1972 small individuals were found in the mouth cavity of the blueback herring. No other *C. carolinensis* were collected on blueback herring, nor on other clupeid fish examined. However, one leech was found in the mouth cavity of the spot, *Leiostomus xanthurus,* and the summer flounder, *Paralichthys dentatus,* and one was collected from the ventral surface of the clearnose skate, *Raja eglanteria,* and the southern flounder, *Paralichthys lethostigma.* Considering that many hundreds of spots and dozens of flounders and rays were examined, it seems likely that these are just accidental relationships of no real significance.

The majority of blood-sucking glossiphoniid and piscicolid leeches leave their hosts and seek a solid substratum during the breeding season and *C. carolinensis* is no exception. In the late winter and early spring months, most of the engorged leeches leave the menhaden and become attached to oyster clumps and associated green seaweeds (*Ulva*) apparently the only suitable substrates commonly available to them in the mud-bottomed tidal creeks of South Carolina. At Beresford Creek on 8 February 1971 and again on 23 February 1972 and for weeks thereafter in each case, large numbers of leeches were dredged up with oyster clumps. It was also about this time, 13 March 1971 and 29 January 1972, that large numbers of engorged leeches were found on detached green seaweeds floating in the surf at Folly Beach.

The rate of digestion of ingested blood is remarkably slow and is evidently very efficient. During digestion the intestine was noticeably much darker than the crop. At 9° C the leeches lived for at least 10 weeks without further feeding, the dark fecal material slowly accumulating on the bottom of the aquaria. All the leeches in the laboratory eventually died.

Like most bloodsucking piscicolids examined, *C. carolinensis* is probably a vector for hemogregarines or hemoflagellates (see Becker and Katz, 1965a and 1965b; and Putz, 1972). Thus, *C. carolinensis* is a probable vector for the hemogregarine, *Haemogregarina brevoortiae,* recently described from menhaden from southern Florida (Saunders, 1964).

Behavior

Locomotion in *C. carolinensis* is in the "inchworm" manner typical of the Piscicolidae. The foresucker is extended until it attaches to the substratum; the hindsucker is then positioned near the midventral portion of the body and slides down the body until it is positioned immediately posterior to the foresucker. When an individual is disturbed, the hindsucker is released and the body coils up like a snake with the head always dorsal. It remains in this position for a number of seconds before it crawls or swims away.

True swimming is strongly developed and can be sustained for periods up to one minute or even longer under ideal conditions. This is done by means of rapid, undulating motions of the flattened body and hindsucker. Swimming can be evoked by jarring the container, by dislodging the hindsucker from the substratum or by suddenly passing a shadow overhead. When the ventral nerve cord is severed, the end posterior to the transection displays swimming motions, whereas the anterior end displays crawling motions. Similarly, when the brain is excised the animal displays swimming motions. Unlike most leeches, *C. carolinensis* does not ventilate its body by undulating the entire body while the hindsucker is attached to the substrate, a manner reminiscent of swimming in position.

In an undisturbed aquarium *C. carolinensis* displays the curious habit of swimming to the surface of the water until the foresucker attaches to the surface film. Almost immediately the hindsucker is then attached and the foresucker is released in such a manner that the animal hangs upside down from the surface film for long periods. They can even crawl upside down along the surface film in the typical manner. This suspending behavior is most common in the smaller individuals (3 to 7 mm); a number of large individuals suspended only once, in a closed container that had been at room temperature for about 24 hours. Individual leeches were collected in plankton nets dragged at the surface of the water on 9 March and 11 March 1971, 3 March 1972, 6 February and 12 February 1973, when the water was about 11 to 12° C.

C. carolinensis displays a strong reaction to a sudden decrease in light intensity. Normally an individual maintained under constant light conditions will quickly respond to a shadow passing overhead with random "searching" motions of the foresucker. In extreme cases the passing shadow will evoke crawling, coiling or even swimming. Individuals which have had their two pairs of eyes removed with a scalpel blade also display the typical "searching" motions in response to a shadow. Unlike many leeches, this species does not display "searching" motions when its container is sharply vibrated. An individual engorged with blood is relatively inactive and usually displays little reaction to a shadow. Leeches maintained in constant dark will barely respond to a sudden light.

Habituation of leeches to a shadow was investigated. Ten leeches were maintained in individual containers at room temperature. The light source was a microscope light positioned about 40 cm from the animals. Experiments were conducted by passing a shadow at intervals of five seconds after the animal became quiescent. The duration of the "searching" response was timed until five negative responses were observed. Stronger responses were observed with the shadow originating at the light source itself rather than originating immediately above the animal. During every experiment the duration of the response gradually decreased to the point that the leech did not respond at all. In the original series of experiments this point varied among individuals from 14 to 109 trials. In a second series of experiments one hour later this point varied from 13 to 25 trials and similar results were obtained in a third series, on a few individuals, 24 hours after the second series of experiments.

The duration of the longest response for each individual varied from 4.0 to 10.0 seconds in the first series of experiments and from 3.8 to 10.0 seconds in the second series. The longest response of each individual occurred in the second to tenth trial in the first series of experiments and in the first to sixth trial in the second series.

C. carolinensis, which has unusually large chromatophores, dramatically demonstrates physiological color change in response to changes in ambient light and temperature (Sawyer and Dierst-Davies, in press). The leeches darken in the light and blanche in the dark, and to a lesser extent darken under warm water temperatures (23.0° C) and blanche under cold water temperatures (9.0° C). The leeches do not adapt to background coloration. It was demonstrated by severing the ventral nerve cord that color response is probably under neurohumoral control from the brain.

Respiration is probably aided by pulsating vesicles which are arranged metamerically along the lateral margins of the body. At 27° C the vesicles pulsated 57 to 64 times per minute, and at 18.5° C 30 to 41 times per minute. However, there is a great deal of variation in the rate of pulsation and there does not appear to be much coordination between the vesicles. When the ventral nerve cord was severed the vesicles at both ends of the body continued to pulsate.

Reproduction and development

Reproduction in *C. carolinensis* involves some interesting aspects which are unique to the Piscicolidae. The anatomy of the unusual reproductive system is described in detail by Sawyer and Chamberlain (1972). Courtship and mating was observed on numerous occasions both in the laboratory and in nature. During courtship two individuals approach one another and make agitated searching motions over one another's body with their foresuckers. At the same time the male bursae of one or both individuals are protruded to form a conspicuous cylindrical penis, a rare structure in the Piscicolidae. From time to time the anterior ends of their bodies tightly intertwine to culminate in true copulation. Copulation in this species involves the introduction of the penis into the spacious bursa of the other individual. It is doubtful whether simultaneous reciprocal copulation takes place in these hermaphrodites. Unlike most other piscicolids that have been studied, two individuals remain *in coitu* for 24 hours or even longer. A series of cross-sections through two individuals fixed *in coitu* revealed that a spermatophore containing sperms is deposited into the unique seminal receptacle. Mating couples face in the same direction or end to end (Fig. 2A) and they frequently vary positions by moving the suckers. When disturbed, the mating individuals often attempt unsuccessfully to pull themselves apart. In fact, it is relatively difficult even to pull them apart manually. Two individuals which had each been tightly ligated with a silk thread as a part of another experiment mated successfully 3½ hours later.

Mating normally takes place within a day of collection and continues with decreasing frequency for many weeks. Mating is encouraged by a change in water, light or temperature conditions. In nature, a mating pair was collected on 29 April 1972 on a piece of floating seaweed. Interestingly, mating was never observed on menhaden but was observed under the mouth of a mullet under laboratory conditions.

Cocoon deposition was observed on numerous occasions in the laboratory (Figs. 2, D–G). The individual places the clitellar region of the body adjacent to the glass substratum for a few minutes before the whitish ring of clitellar secretions become evident (Fig. 2D). When the ventral portion of this sticky ring firmly adheres to the glass, the body rotates itself within the ring, presumably to free the ring from the body wall (Fig. 2E). After a minute or so of rotation, the body anterior to the ring elongates (Fig. 2F) tremendously in preparation for the swift posterior movement which pulls the body through the ring (Fig. 2G). Unlike many leeches, *C. carolinensis* gives no further care to the helmet-shaped (diameter, 0.5 mm) cocoons (Figs. 2, B–C) which harden and become dark

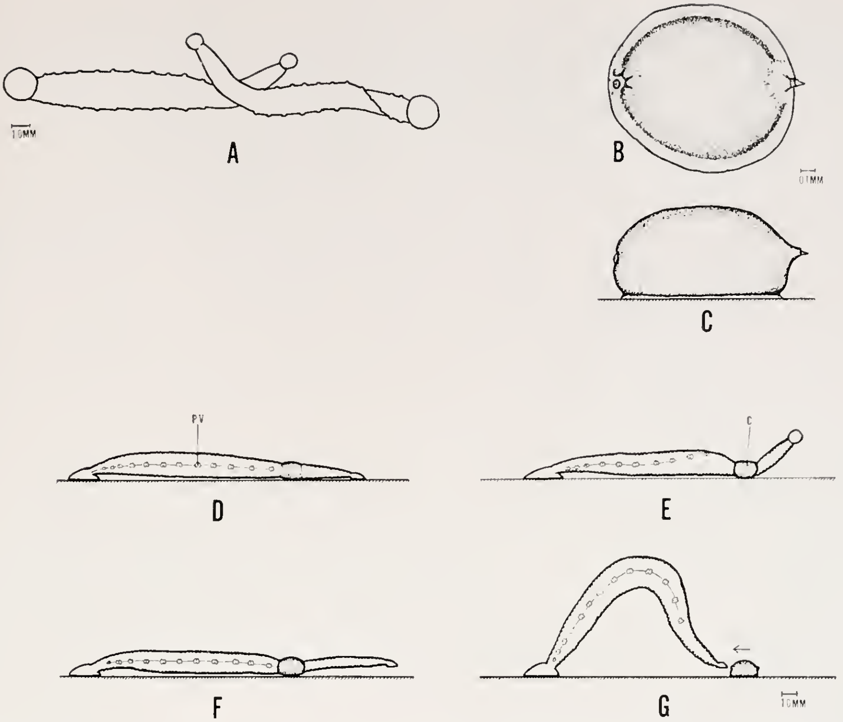

FIGURE 2. *Calliobdella carolinensis;* A, two copulating individuals; B, dorsal view of cocoon; C, lateral view of cocoon; D–G, cocoon deposition. See text for further explanation.

brown in about an hour. Like the cocoons of all other Piscicolidae, each cocoon contains only a single egg.

Throughout cocoon deposition, which takes 3 to 4 minutes, the hindsucker is firmly attached, whereas the foresucker is free to move about. The individual usually remains attached to the same spot and deposits many more cocoons. Often the deposition of cocoons is interrupted by mating, again without moving the hindsucker. Sometimes the individual moves to several other spots to deposit the rest of the cocoons.

In the laboratory the first cocoons were deposited on 12 February 1971 and 26 February 1972 by individuals collected on 8 February 1971 and 23 February 1972 when the water temperature was 9.2° and 9.4° C, respectively. Fourteen individuals collected on 8 February 1971 first mated and deposited cocoons on 12 February. Most of the cocoons were deposited between 15 and 19 February, and the last of a total 211 cocoons was deposited on 24 February. Most of the cocoons were aggregated into ten clumps of 7 to 29 cocoons. Assuming that 10 individuals were responsible for the aggregations, 21.1 cocoons per individual can be conservatively estimated. Similarly, two leeches collected on 5 March 1973 shortly afterwards deposited 41 cocoons.

In spite of repeated attempts, no cocoons were ever found in nature, but they will probably be found on the green seaweed *Ulva* or on oyster shells, the only common solid substrates suitable for cocoon deposition in the mud-bottomed tidal creeks. In the laboratory cocoons deposited on 14 February 1973 on oyster shells and on the walls of the aquarium hatched nine weeks later (water 21.5° C). In April and May 1971 and 1972, similar, but smaller, helmet-shaped cocoons of the swimming marine triclad, *Bdelloura rustica* Verrill, were frequently encountered on *Ulva* and at first confused with the cocoons of *C. carolinensis*. In laboratory containers maintained at 9° C the leech cocoons failed to hatch each year by May, at which time they were discarded. The leeches are about 3.0 mm long when they hatch from cocoons. The immature individuals (3 to 9 mm) differ only slightly from the mature individuals (9 to 29 mm) (Sawyer and Chamberlain, 1972). The former are more cylindrical with no distinction between the urosome and the trachelosome. Moreover, the body walls of the immature forms are usually translucent white, becoming more darkly pigmented with age. In the smallest individuals the lateral vesicles are small and internal, and the coelomic system is somewhat reduced. Almost identical differences between the immature and mature individuals were noted in the marine leech *Oceanobdella blennii* by Sawyer (1970).

Apart from the study by Sawyer (1970), the problem of growth in leeches has received very little attention. An analysis of growth patterns in *C. carolinensis* was undertaken on a population of 91 individuals ranging in size from 5 to 25 mm, collected on 13 March 1971 at Folly Beach. For each individual, which had been relaxed with 70% ETOH and fixed with formalin, the total length (L) of the body, the maximum width (W) of the body, and the maximum widths of the foresucker (F) and the hindsucker (H) were measured with an ocular micrometer. The data were analyzed for variance and non-linear regression with the aid of a digital computer. The analysis demonstrates clearly that, whereas some parts of the body grow at a faster rate than others, the growth of the four measured parts of *C. carolinensis* was essentially linear over the size range 5 to 25 mm (Table I).

Reconstruction of the life cycle of Calliobdella carolinensis

C. carolinensis normally lives in the mouth cavities of the Atlantic menhaden from mid-December to January. Starting in early January, when the water temperature decreases to about 12 to 13° C, the mature (9 mm and larger) leeches begin to leave the host in large numbers to reproduce in the tidal estuaries. Each individual deposits at least 20 cocoons in middle February when the water temperature is 9 to 10° C. Cocoons are probably deposited on oyster clumps or other solid substrates. Adults die by May, and no leeches are found until the following mid-December or early January when the young attach to juvenile (100 to 104 mm, SL) menhaden which have overwintered in estuaries. The newly emerged young are about 3.0 mm long and are good swimmers. Some probably find their hosts, which feed near the surface, by suspending themselves upside down on the surface film of the water. Others probably find their hosts by swimming toward a school of menhaden as it casts a shadow on the bottom.

DISCUSSION

The epizootic outbreak of *C. carolinensis* reported above for January–March 1971 and 1972 is probably attributable to some factor(s) responsible for the in-

TABLE I

Regression analysis of growth in a population of 91 (one discarded) specimens of Calliobdella carolinensis *collected 13 March 1971 from Folly Beach. L, F, H and W mean total body length inclusive of both suckers, foresucker width, hindsucker width, and maximum body width, respectively. LW indicates an analysis of the relationship between the body total length and its maximum width,* etc.

	LW	LF	LH	WF	WH	FH
Regression coefficient	0.06718	0.03857	0.007440	0.39532	0.61057	1.54915
Standard error of regression coefficient	0.00623	0.00283	0.00582	0.03699	0.08932	0.12988
Correlation coefficient	0.75454	0.82346	0.80607	0.75150	0.58894	0.78605
Analysis of variance for simple linear regression (F value)	116.334	185.364	163.254	114.186	46.731	142.294
Sample size	90	90	90	90	90	90

creased numbers of survival of the offspring laid in January–March 1970 (or even one year prior). It now seems likely that an unusually cold spell and a high level of turbidity may have played an important role in this epidemic. As part of another study, surface water temperatures were recorded for every month from 1963 to 1972, at several routine sampling stations near Charleston. The coldest temperatures, which normally run 9.0–10.0° C, occur each year in January and February. Unfortunately, due to administrative changeovers no recordings were taken during the crucial period of January 1970. However, the records of the U. S. Weather Bureau at Charleston show that the monthly average air temperature of January 1970 (5° C) was the coldest for any month of any year since January 1940. This was well below the average January monthly air temperature of 10° C in the Charleston area. Unusually cold water temperatures were also recorded at all stations in February 1966 (6.8–7.2° C) and in January 1968 (3.5–4.8° C).

A related factor that may be overlooked is the high level of silt in the highly turbid (Secchi disk typically visible at 38 to 45 cm) waters of the estuaries of the Charleston area. This silt may have increased the turbidity of the water to such a level that, along with decreased water temperatures, natural predation on *C. carolinensis,* especially on the free-living breeding adults, was drastically reduced. The pollution ecology of leeches has been discussed in some detail elsewhere by Sawyer (1973) who presents experimental evidence supporting the supposition that natural predation by fish on leeches is decreased in turbid water. That external commensals and parasites are indicators of silt pollution receives circumstantial support from other species in this area. For example, the population levels of the brackish water leech, *Illinobdella moorei* Meyer, found on the white catfish, *Ictalurus catus* (Linnaeus); the marine leech, *Myzobdella lugubris* Leidy, found on the blue crab, *Callinectes sapidus* Rathbun; and the marine triclad, *Bdelloura rustica* Verrill, are much higher in this area than in similar regions of South Carolina where the water is much clearer.

Among freshwater leeches epizootic outbreaks are not unknown. Richardson (1928) presented a well-documented description of an outbreak of leeches (especially of *Helobdella stagnalis*) in the Middle Illinois River in 1925. In the region

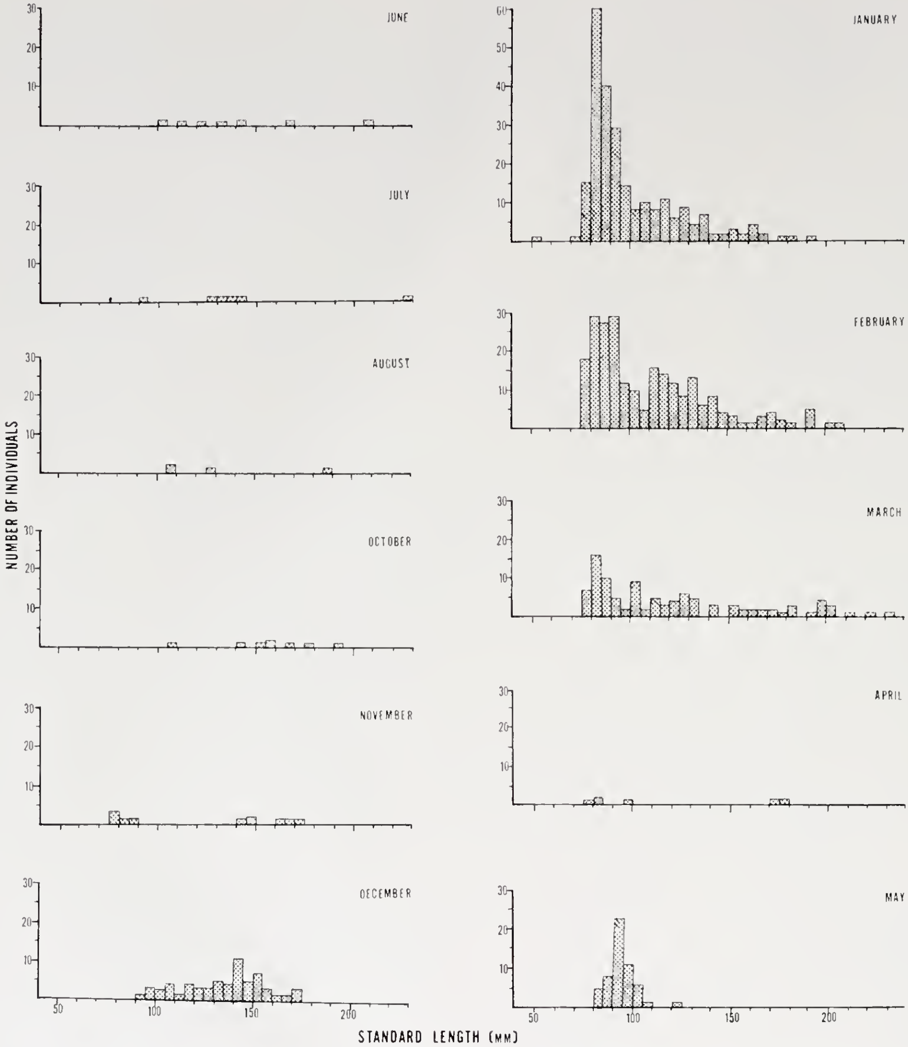

Figure 3. The relative abundance, seasonal occurrence and length frequency distribution (SL) of the Atlantic menhaden (*Brevoortia tyrannus*) collected by the South Carolina Marine Resources Center from Georgetown south to Hilton Head Island, S. C., for each month (excluding September) of 1970 and 1971 (combined data).

of the river most affected, the number increased from 1985 individuals per square meter in 1924 to as high as 29,107 in 1925. The total weight of the leeches changed from just over 200 kilograms per hectare (180 lb/acre) in 1924 to over 2800 kilograms per hectare (2550 lb/acre) in 1925. This meteoric rise in the concentration of leeches was undoubtedly due partially to the eutrophication of the river at that time. A similar outbreak of the fish leech *Piscicola punctata* was

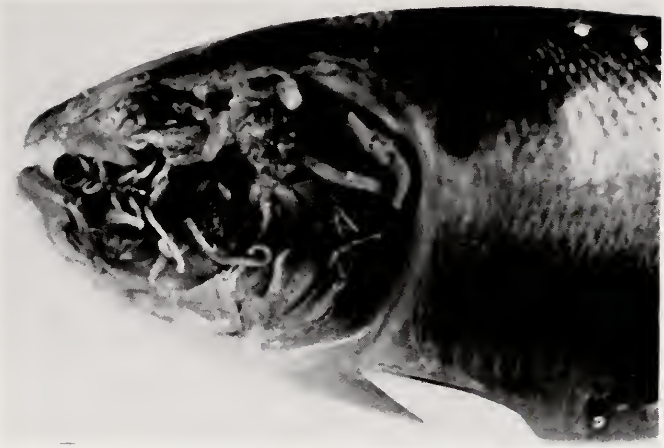

FIGURE 4. A specimen of *Brevoortia tyrannus* 155 mm, SL, collected near Charleston, S. C., 14 January 1971 displays 21 specimens of *C. carolinensis* and one isopod in the oral cavity.

described by Thompson (1927) in the nearby Rock River during the winter of 1925–1926. The course of this outbreak of *P. punctata,* a near relative of *C. carolinensis,* was remarkably similar to that described here for *C. carolinensis.*

One intriguing aspect of the life cycles of many piscicolid leeches is the conspicuous long absence of the leeches from the area soon after the adults leave the host to deposit their cocoons. For example, Halvorsen (1971) working with *Cystobranchus mammillatus,* Hoffman (1955) with *Cytobranchus respirans,* Thompson (1927) with *Piscicola punctata,* Becker and Katz (1965b) with *P. salmositica,* Halvorsen (1972) with *P. geometra,* Gibson and Tong (1969) with *Oceanobdella blennii,* and now this study with *C. carolinensis* all report intervals, usually from early summer to early winter, in which leeches are very scarce or are not to be found anywhere. The general consensus has been that the development or growth of the immature leeches is retarded during this period. Although our studies have shown that *C. carolinensis* can hatch in the laboratory after 9 weeks (water 21.5° C), no leeches were found in nature until the following winter. On 13–14 December 1972 (water 15.0 and 16.8° C, respectively) small individuals (6–10 mm) of *C. carolinensis* were found at Beresford Creek and elsewhere, in the mouth cavity of the blueback herring, *Alosa aestivalis,* as well as on menhaden. These individuals support the supposition that growth in this species is retarded until about mid-December.

Our investigations suggest that the life history of *C. carolinensis* is intimately associated with that of its host, the Atlantic menhaden. Unfortunately, very little is known about the ecology, population dynamics and life history of the menhaden in the numerous estuaries of South Carolina although extensive work in other portions of its range has been carried out by such investigators as June and Chamberlain (1958), June and Carlson (1971), McHugh, Oglesby and Pacheco (1959), Reintjes (1969), Wilkens and Lewis (1971) and Nicholson (1972). Briefly, these workers agree that the Atlantic menhaden ranges from central

Florida to New England. In the southern portion of its range, the adult menhaden spawns offshore during the winter. The larval fish (8 to 40 mm, TL) enter the estuaries after the yolk sac has been absorbed, moving up the estuaries to the freshwater transition zone, where they begin the transformation to juveniles. Becoming juveniles at about 113 mm (50 to 135 mm, SL), they form schools and enter all areas of the estuary, filter-feeding on plankton. They normally spend six to eight months in the estuaries before returning to the ocean in the autumn. A portion of the juvenile population will spend the winter in the nursery grounds, leaving the following year when they have attained approximately 179 mm, SL.

Based on data compiled by the South Carolina Marine Resources Center in routine monthly samples of menhaden caught in otter trawls in 1970 and 1971, the menhaden in the estuaries near Charleston have a definite seasonal occurrence (Fig. 3). They were most abundant in collections made in January, February and March. After February there was a monthly decline in the numbers caught in the trawls. The modes (and ranges) of the standard lengths of the individuals caught in the months of Febuary, April, June, September and December, 1970 and 1971 (combined data), were 90 (55–172), 94 (68–167), 113 (56–154), 115 (94–180) and 90 (62–145) mm, respectively. The abundance of menhaden in the winter months possibly reflects diminished net avoidance due to the low temperature of the water. *Calliobdella carolinensis* was especially prominent during this period. In our investigations *C. carolinensis* was found on menhaden ranging from 91 to 277 mm, SL, with most occurring on fish of 100 to 104 mm, SL (Fig. 4). Presumably these juveniles spent the winter in the estuaries where they became infected with the immature leeches.

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SUMMARY

1. In January through March, 1971, and to a less extent the same period in 1972 and 1973, the marine leech, *Calliobdella carolinensis* Sawyer and Chamberlain 1972, was epizootic on the Atlantic menhaden, *Brevoortia tyrannus*, in the estuaries of South Carolina. Prior to this epizootic outbreak (epidemic) the leech was practically unknown.

2. The population dynamics of this outbreak were studied near Charleston, South Carolina, for three years, primarily in an estuarine creek and in the surf zone of an exposed beach.

3. *Calliobdella carolinensis* displays a seasonal occurrence which correlates with the temperature of the water. The leeches reach their greatest abundance in late February and early March, when the water is coldest (9 to 10° C). At that time the leeches breed and deposit at least 20 helmet-shaped cocoons. No leeches are found from the end of April until the following mid-December.

4. The leech occurs primarily in the estuaries of South Carolina, and is reported also from Virginia. It is not known south of South Carolina.

5. Feeding, reproductive behavior, growth, life cycle, salinity tolerance and other aspects of the biology of *C. carolinensis* are described.

LITERATURE CITED

Becker, B. D., and M. Katz, 1965a. Distribution, ecology and biology of the salmonid leech, *Piscicola salmositica* (Rhynchobdellae: Piscicolidae). *J. Fish. Res. Board Can.,* **22**(5): 1175–1195.

Becker, C. D., and M. Katz, 1965b. Transmission of the hemoflagellate, *Cryptobia salmositica* Katz, 1951, by a rhynchobdellid vector. *J. Parasitol.,* **51**: 95–99.

Gibson, R. N., and L. J. Tong, 1969. Observations on the biology of the marine leech *Oceanobdella blennii. J. Mar. Biol. Ass. U. K.,* **49**: 433–438.

Halvorsen, O., 1971. Studies of the helminth fauna of Norway, XIX. The seasonal cycle and microhabitat preference of the leech, *Cystobranchus mammillatus* (Malm 1863) parasitizing burbot, *Lota lota* (L.). *Norwegian J. Zool.,* **19**: 177–180.

Halvorsen, O., 1972. Studies of the helminth fauna of Norway. XXX. Seasonal cycles of fish parasites in the River Glomma. *Norwegian J. Zool.,* **20**: 9–18.

Hoffman, J., 1955. Quelque charactéres éthologiques de la Piscicolidée: *Cystobranchus respirans* Troschel. *Arch. Inst. Gr.-Duc. Luxemb. N.S.,* **22**: 223–225.

June, F. C., and J. L. Chamberlain, 1958. The role of the estuary in the life history and biology of Atlantic menhaden. *Proc. Gulf Carib. Fish. Inst.,* **11**: 41–45.

June, F. C., and F. T. Carlson, 1971. Food of young Atlantic menhaden, *Brevoortia tyrannus,* in relation to metamorphosis. *U. S. Fish Wildlife Serv. Fish. Bull.,* **68**(3): 493–512.

Llewellyn, L. C., 1965. Some aspects of the biology of the marine leech *Hemibdella soleae. Proc. Zool. Soc. London,* **145**(4): 509–528.

McHugh, J. L., R. T. Oglesby and A. L. Pacheco, 1959. Length, weight and age composition of the menhaden catch in Virginia waters. *Limnol. Oceanog.,* **4**(2): 145–162.

Nicholson, William R., 1972. Population structure and movements of Atlantic menhaden, *Brevoortia tyrannus,* as inferred from back-calculated length frequencies. *Chesapeake Sci.,* **13**(3): 161–174.

Putz, R. E., 1972. *Cryptobia cataractae* sp.n. (Kinetoplastida: Cryptobiidae), a haemoflagellate of some cyprinid fishes of West Virginia. *Proc. Helm. Soc. Washington,* **39**(1): 18–22.

Reintjes, J. W., 1969. Synopsis of biological data on the Atlantic menhaden (*Brevoortia tyrannus*). *U. S. Fish Wildlife Serv., Circ.* **320**: 1–30.

Richardson, R. E., 1928. The bottom fauna of the Middle Illinois River, 1913–25. Its distribution, abundance, valuation, and index value in the study of stream pollution. *Bull. Ill. Natur. Hist. Surv.,* **17**: 387–475.

Saunders, D. C., 1964. Blood parasites of marine fish of southwest Florida, including a new haemogregarine from the menhaden, *Brevoortia tyrannus* (Latrobe). *Trans. Amer. Microscop. Soc.,* **83**: 218–225.

Selensky, W. D., 1923. *Crangonobdella murmanica* n.g. n.sp., eine auf *Sclerocrangon* schmarotzende Ichthyobdellide. *Zool. Jahrb. Abt. Syst. Geo. Biol. Tiere,* **46**: 397–488.

Sawyer, R. T., 1970. The juvenile anatomy and post-hatching development of the marine leech, *Oceanobdella blennii* (Knight-Jones, 1940). *J. Natur. Hist.*, **4**: 175–188.

Sawyer, R. T., 1973. Pollution ecology of freshwater leeches. In press in C. W. Hart, Jr. and S. L. H. Fuller, Eds., *Pollution Ecology of Freshwater Organisms.* Academic Press, New York.

Sawyer, R. T., and N. A. Chamberlain, 1972. A new species of marine leech (Annelida: Hirudinea) parasitic on the Atlantic menhaden, *Brevoortia tyrannus. Biol. Bull.*, **142**: 470–479.

Sawyer, R. T., and K. Dierst-Davies, 1973. The physiology and phylogeny of colour change in freshwater and marine leeches (Annelida: Hirudinea). *Hydrobiologia,* in press.

Thompson, David H., 1927. An epidemic of leeches on fishes in Rock River. *Bull. Ill. State Natur. Hist. Surv.*, **17**: 195–201.

Wilkens, E. P. H., and R. M. Lewis, 1971. Abundance and distribution of young Atlantic menhaden, *Brevoortia tyrannus,* in the White Oak River Estuary, North Carolina. *U. S. Fish. Wildlife Serv. Fish. Bull.*, **69**(4): 783–789.

Reference: *Biol. Bull.*, **145**: 389–422. (October, 1973)

PHYSIOLOGICAL ECOLOGY AND INTERTIDAL ZONATION IN LIMPETS (*ACMAEA*): A CRITICAL LOOK AT "LIMITING FACTORS"[1]

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The striking intertidal gradient from marine to terrestrial physical conditions is correlated with an equally obvious spatial partitioning of the habitat by groups of animals. This has repeatedly given rise to the assumption that terrestrial physical factors, acting during tidal emersion, determine the upper range limits of intertidal populations. This assumption is elevated to the level of a hypothesis in this study and tested with scientific rigor. If conditions in the physical environment serve as "limiting factors," determining the partitioning of the intertidal, it should be demonstrable that (a) interspecific differences in physiological tolerances exist and permit differential exploitation of the habitat, and (b) conditions in the microenvironment of the animals exceed the physiological tolerances at the fringes of ranges and, by causing mortality, prevent range extension.

The conditions most often assumed to serve as "limiting factors" are high temperatures (Huntsman and Sparks, 1924; Broekhuysen, 1940; Allanson, 1958; Read and Cumming, 1967; and many others) and/or desiccation (Test, 1945; Shotwell, 1950b; Haven, 1970). The assumption rests on two sorts of correlations: those between physiological tolerances and zonation, and those between fair weather and death or disappearance of animals in the field.

A relationship between laboratory physiological tolerances and zonation is probably the most frequently demonstrated facet of intertidal biology. Numerous workers have shown that, in comparison with organisms living lower, high-intertidal animals tend to have higher lethal temperatures (Evans, 1948; Southward, 1958; Fraenkel, 1968; Sandison, 1968; Hardin, 1968; Davies, 1970), higher desiccation tolerances and lower desiccation rates (Colgan, 1910; Broekhuysen, 1940; Allanson, 1958; Brown, 1960; Bock and Johnson, 1967), and greater tolerance of osmotic extremes (Broekhuysen, 1940; Arnold, 1957; Brown 1960; Davies, 1969). The high tolerances of high-intertidal animals, and some of the physiological effects of exposure, have been especially well documented in the careful and detailed work of Kensler (1967) with crevice-dwelling invertebrates, Foster (1971) with barnacles, and Davies (1969, 1970) with British limpets (*Patella*). Unfortunately, even in these studies there is a paucity of data concerning the extremes of the microclimates experienced by the animals in the field. Without a demonstration that physical conditions in the field exceed the physiological tolerances of the animals, it cannot be stated with confidence that physical factors are limiting. In actuality, in those studies where field measurements are given, body temperatures (Southward, 1958; Hardin, 1968; Davies, 1970; Markel, 1971), and desiccation level or osmotic stress (Segal and Dehnel, 1962; Davies, 1969; Boyle, 1970), are always well below the lethal limits as determined in the laboratory.

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Occasionally, observers have noted the death or disappearance of animals from the upper ends of intertidal ranges, usually during warm, dry weather, often when midday low tides coincided with minimal wave splash (Orton, 1933; Hodgkin, 1959; Frank, 1965a; Sutherland, 1970). However, since no measurements of field microclimates or condition of animals were made at the time of these "kills," again it is impossible to establish a casual relationship between physical conditions and the proximal cause of death. For example, the finding of dead, dry animals in the field can mean either that the animals were killed by desiccation, or that the animals dried out because they were dead.

In short, despite countless studies showing how intertidal animals are adapted to live where they do, the actual importance of physical factors in limiting the distribution of populations remains unclear. To shed additional light on this issue, an intensive study has been made of the physiological ecology and zonation of five species of limpets (*Acmaea*) from the central California coast. All five species are similar in their morphology and basic biology, but occupy different zones and microhabitats; thus they present a convenient system in which to compare the effects of physical factors. The hypothesis that the upper limits of their distributions are determined largely by physical environmental factors has been tested according to the criteria mentioned earlier. Laboratory tolerances to the environmental conditions likely to produce stress in the field have been measured, to determine if interspecific differences allow exploitation of different portions of the intertidal gradient. Concurrently, the extremes of the same environmental conditions occurring in the microhabitats of each species have been monitored for a period of three years, to determine if these conditions ever exceed the tolerances of the limpets and, by causing mortality, limit their ranges.

Materials and Methods

The five species of limpets used all occur commonly on rocky central California shores. Three of the species normally inhabit the bare rocks of the splash zone (Zone I of Ricketts and Calvin, 1962)—from about 5 feet above mean lower low water (MLLW) to the limit of high-water splash. *Acmaea digitalis* Eschscholtz, 1833, is a small (1–2 g) greenish-gray to eroded brown limpet occupying primarily vertical or overhanging surfaces (Haven, 1970). On cliffs receiving large amounts of spray this species ranges higher than any other, up to 30 feet above MLLW. *Acmaea scabra* (Gould, 1846) is a small (1–2 g) heavily ribbed gray to white limpet occupying primarily horizontal surfaces fully exposed to the sun (Haven, 1970). It returns regularly to the same orientation on the same scar on the rock surface, or "homes," and the shell margin grows to fit the home scar precisely. *Acmaea persona* Eschscholtz, 1833, is a large (to 10 g), smooth olive-shelled limpet, found in dark crannies and under boulders, sheltered from the sun. It is negatively phototactic, moving and feeding only during the night (Richardson, 1934; Ricketts and Calvin, 1962).

Two of the species are found among the scattered clumps of algae in the upper mid-tidal (Zone II of Ricketts and Calvin, 1962)—from about +4 to about +5 feet above MLLW at Bodega Head. *Acmaea pelta* Eschscholtz, 1833, is a high-peaked brown limpet, very variable in form, which typically reaches a maximum size of about 5 g. It is characteristically found among or under macroscopic algae on surfaces experiencing mild to moderate wave action, such as the walls of surge channels. *Acmaea testudinalis scutum* Eschscholtz, 1833, is a moderate-sized (2–4 g) limpet distinguished from the others by its extremely flat shell profile. It is characteristically found on the damp, shaded walls or boulders at the bottom of surge channels, or in tidepools.

The study was carried out at the University of California's Bodega Marine Laboratory, located on Bodega Head, Sonoma County, California, about 110 km north of San Francisco. The rocky shore at the Laboratory is composed of heavily jointed, extremely rugged diorite granite. The biotic zonation is in accord with the descriptions of Ricketts and Calvin (1962) for exposed rocky coast, though modified by wave action and the unevenness of the surface.

The weather is dominated by the cold Japanese current into which Bodega Head juts; water surface temperatures range from 9° C in May to 16° C in September. Mean daily maximum air temperatures range from about 10° C in January to about 20° C in late August; very rarely mild frosts occur on winter nights. Aside from winter rainstorms, bringing from 45 to 125 cm (average 76 cm) of rain per year, two principal types of weather occur at the Laboratory: considerable periods of fog and little wind, especially during the summer; and periods of sunny weather with high (30–100 km/hr) northwest winds. Occasionally there are days that are both clear and calm; rarely (probably less than 10 days each year) these conditions will be accompanied by air temperatures exceeding 20° C (based on local meterological data analyzed by M. G. Barbour (University of California, Davis) and K. L. van der Laan (University of California, Berkeley); personal communication).

The tides at Bodega Head are of the mixed semi-diurnal type, with two unequal high and two unequal low tides each day. During the spring and early summer the lower low tide occurs during the morning hours and is followed by the lower high tide. At such times both Zone I and Zone II may remain exposed all day, being submerged only a few hours during the night. During these periods, maximum stresses are experienced by intertidal organisms, particularly if midday low tides occur on one of the rare warm days. Time of day of lower low water and expected submergence times through the year at the Bodega Marine Laboratory have been calculated by Sutherland (1970). When seas are calm, Zone I limpets may expect a maximum of 4 hours immersion once per day and a minimum of no immersion for several consecutive days during portions of the spring and early summer. In contrast, Zone II limpets may expect a minimum of 4 hours immersion at least once per day during these same periods.

Limpets were collected from rocky shores near the Laboratory, and as far north as the Russian River, about 13 km away. Capture was effected by a quick prying motion with the "limpet snatcher," a sawed off stainless steel table knife.

Those individuals which did not yield to the first effort were left, since experience showed that products of the second and subsequent tries were usually injured and unusable. Limpets were returned to the laboratory and placed under running sea water immediately after collection. All animals were used within 48 hours, with the exception of some *A. scabra,* which were maintained for several weeks on glass plates in a system providing two artificial tides of fresh sea water daily. Some food was provided in the form of microscopic algae growing on the plates. This regime permitted the growth of flat shell margins on *A. scabra,* which facilitated comparison with the other species in desiccation studies.

Field measurements were designed to evaluate the extremes of microclimates experienced by, or available to, the limpets. Measurement sites were selected accordingly; for instance, the majority of high-temperature data was collected in Zone 1 on the south-facing side of the cove adjacent to the Laboratory, between 12:00 and 15:00 on clear, calm, exceptionally warm days. During the relatively few hours of such hot periods, intensive searches were made for limpet microhabitats that received maximum solar radiation and were sheltered from wind and splash.

Temperatures of air (shaded) at 2 m and 1 cm above the rocks, rock surface temperatures, and limpet body temperatures were measured by two methods. Where portability was a prime concern, a Yellow Springs Instruments Telethermometer was used with a type 506 (22 gauge) hypodermic needle thermistor probe calibrated against a standard laboratory thermometer. Limpet temperatures were obtained by prying the animals off the rock, quickly inserting the needle probe into the center of the visceral mass, and immediately pressing the limpet onto its original location with a fingernail or pencil to minimize heat gain or loss. The maximum temperature indicated was recorded. This method required sacrificing a limpet for each temperature determination. Since body temperatures of limpets were virtually the same as temperatures of the adjacent rock surfaces (mean deviation of 12 specimens of *A. scabra* from rocks was −0.33° C ± 0.19 (S.E.) at 15:00, 8 August 1971), in many cases only rock surface temperatures were taken rather than depopulating the research area of limpets.

Where recordings of temperature fluctuations over an entire day were desired, a 12-channel Leeds-Northrup Speedomax thermocouple recorder was used, powered by a portable 115 V generator. Size 7 (#36 gauge) thermocouples were fastened to rock surfaces with lumps of modelling clay placed about 5 cm from the tip of the couple. Recordings of limpet body temperatures could also be obtained, without harm to the limpets, by slipping the tip of the thermocouple under the edge of the shell and fastening the leads down as above.

Interspecific differences in maximum temperatures attained in the field were examined by selecting a study area in which all five species occurred, and recording the temperatures of the individuals of each species which appeared most exposed to solar radiation.

Windspeeds were measured with a Hastings-Raydist portable heated-thermocouple anemometer with an omnidirectional probe which allowed measurement of air movements 2 cm from the rock surface. Measurements were also taken at greater distances from the surface to permit comparison of microclimatic conditions with traditional macroclimatic readings.

Relative humidities were measured with a Hygrodynamics, Inc. electric hygrometer indicator. The probe was placed on the rock surface near the groups of limpets, shaded, and allowed to equilibrate with the air just above the surface. Humidity of air a meter above the surface was also measured.

Thermal tolerances of the five species were compared in the laboratory by a technique similar to that of Fraenkel (1968), which ensured that each individual received a similar heat dose. Tolerances in preliminary experiments were very consistent within species; small sample sizes were therefore used to both minimize cooling of the test bath by a large mass of cold limpets and to avoid needless destruction of animals. Six to ten limpets were allowed to adhere to the walls of a 500 ml beaker. The cold sea water in the beaker was then replaced with aerated sea water at the desired test temperature and the beaker placed in a water bath at the test temperature for 15 minutes. At the conclusion of the experiment the warm water was replaced with running sea water for recovery and assessment of survival. For each species the thermal lethal limit was defined as the lowest temperature killing more than half of the sample tested.

Survival at the conclusion of this and subsequent experiments was assessed after a 24-hour recovery period in running sea water. The criterion of survival was the resumption of normal locomotion, including the ability to adhere to and move about on an inverted glass surface. Animals incapable of normal locomotion, even if responsive to prodding or capable of some spontaneous movement, were considered "ecologically dead" since in the field they would undoubtedly be swept off the rock by surf and perish.

Most thermal tolerances were determined with limpets collected during the summer. Variation of these tolerances throughout the year, and in individuals from different intertidal heights, was checked by collecting *A. digitalis* and *A. scabra* from Zones I and II in winter and determining thermal tolerances during immersion as above.

To explore the relationship between thermal tolerances during immersion and those pertaining in the field, lethal limits were also determined in air with a slow temperature rise which would result in a heat dose equal to or greater than that which the limpets would experience in nature. Ten specimens of *A. digitalis*, *A. pelta*, or *A. scutum* were placed in each of several beakers and allowed to adhere to the walls as above. *Acmaea scabra* was omitted due to its poor fit to the smooth glass, and the consequent problem of desiccation; *A. persona* was omitted due to scarcity. The water was emptied out of the beakers and a few hours allowed for the surface to dry. The beakers were then placed in a recirculating wind tunnel with slow (0.5 m/sec) air movement, and the temperature of the limpets (monitored via thermocouples) was raised over a period of 5–6 hours to the lowest desired test temperature, as would naturally occur with limpets exposed by a morning tide. After 15 minutes at this temperature, the first sample of limpets was removed to be cooled and tested for survival. The temperature was raised 1° C and held for about 15 minutes, another sample removed, and so forth. Effect of size and desiccation state on thermal tolerance under these simulated natural conditions was assessed by including samples of very small and of moderately desiccated *A. digitalis*.

Determination of desiccation tolerances and rates required weighing, observation, or similar manipulations of limpets without disturbing them and causing adventitious water loss from the mantle cavity. These operations were carried out with the animals attached to discs of transparent 0.004 inch Mylar plastic film. Attachment was effected by immobilizing the limpets, shell downward, on lumps of modelling clay on a water table and presenting the disc to the foot. Healthy limpets attached securely to the disc and became quiescent after several hours or overnight on the water table.

Desiccation rates were always determined with moving air, since still air is not a natural situation. Furthermore, air movement is of such crucial importance to evaporation rates that the difference between truly still air and slight convection currents of unknown magnitude would introduce unacceptable uncertainty into measurements of desiccation rates. The use of moving air of constant velocity unfortunately precludes comparison with data of other workers who have used nominally still air (*e.g.*, Davies, 1969).

Progress of desiccation was followed by periodic weighings. Dry, shell and disc weights obtained after termination of the experiments allowed calculation of water lost at any time as a percentage of the initial gross weight, or as a percentage of the total initial body water (wet gross weight—dry gross weight). Because of variation in the shell component of gross weight, the latter figure is considered to be the most meaningful biologically and is the one used for most comparisons; the former allowed approximations where the animals could not be sacrificed for dry weights.

Desiccation rate studies in the laboratory were carried out in a closed, recirculating wind tunnel within a constant-temperature enclosure, with humidity controlled by pans of appropriate salt solutions (*Handbook of Chemistry and Physics*, Chemical Rubber Co., 1958).

Drying of limpets prior to determination of desiccation tolerances took place at 20° C and at 1.0–1.4 m/sec airflow. The humidity was that of the outside air drawn into the ventilating system (usually 50–80% R.H.). These conditions approximate those of a breezy, overcast day. Desiccation was begun after recording of initial weights. Then, several times each day, those animals which appeared near death were removed, weighed again, and checked for survival; dry weights were subsequently obtained. The lowest desiccation levels causing 50% or higher mortality in samples of each species were designated the desiccation tolerance limits.

Tolerance of *A. t. scutum* was also determined under less severe drying conditions to check the effects of rate of desiccation on desiccation tolerance.

Survival at the conclusion of these experiments was assessed as described above.

Since initial hydrated weights of limpets in the field were not available, the weighing method could not be used for field measurements of desiccation. Therefore, a graph was prepared from laboratory data comparing water loss, measured gravimetrically, with chloride concentration in mantle-cavity water/urine ("extra-corporeal water") of 78 specimens of *A. digitalis*. The chloride determinations were carried out on 1 microliter samples of fluid with a Buchler-Cotlove Chloridometer. The resulting curve (Fig. 1) was used to translate chloride concentrations of extra-corporeal water obtained from animals in the field into per cent body water lost at the time the sample was collected.

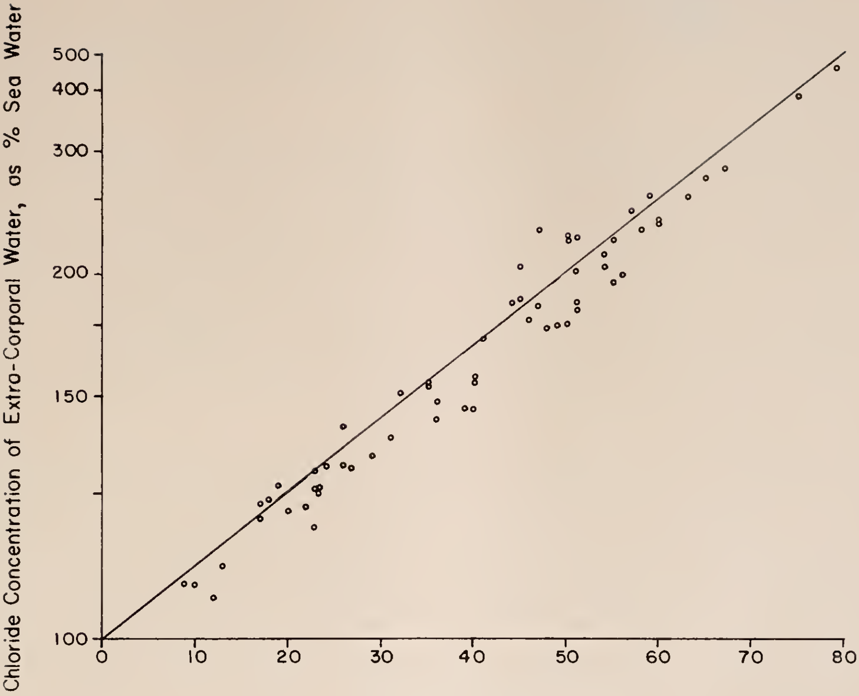

FIGURE 1. Evaporative concentration during desiccation in *Acmaea digitalis*. Line represents values calculated for evaporation of water from an ideal salt solution. To linearize data, vertical scale is proportional to 1 − (100/concentration).

Equilibration rates and tolerances to fresh water were determined by allowing groups of limpets to attach to plastic film as described above, immersing them in fresh water at 15° C, and sampling the population at intervals. Since flow rates profoundly influence boundary layers and diffusion rates, still water was used to provide identical, although somewhat unnatural, conditions for all limpets. Animals removed from the fresh water were allowed about two hours in still air to equilibrate body fluids with the mantle cavity water and urine ("extra-corporeal water" or E.C.W.). The "extra-corporeal water" was sampled via a capillary micropipette after removing the limpet from the substrate, and if necessary, gently pressing the foot. No distinction was made between mantle-cavity fluid and urine because there appears to be no osmoregulation between them (Webber and Dehnel, 1968). Chloride concentration was determined with a Buchler-Cotlove chloridometer. The animals were subsequently assessed for survival.

Comparison of concentrations of blood and E.C.W. in freshwater stressed animals was made both with the Chloridometer and with a nanoliter freezing-point osmometer (Clifton Technical Physics). Blood samples were obtained after all extra-corporeal water had been expressed and blotted away. The foot was slit and the blood that seeped into the cut was then drawn up in a capillary micropipette.

Pressure on the foot usually aided this process. Samples for osmometry were kept deep-frozen under oil until processed. For ease of comparison, all concentrations are expressed as per cent sea water, where 100% sea water has a salinity of 35‰.

Tolerance to acute immersion in solutions hyperosmotic to sea water was tested by immersing limpets for 5 hours in aerated solutions, followed by return to running sea water for subsequent assessment of survival. Test solutions were made up from sea water and stock 500%–700% sea water prepared by boiling under vacuum, or with "Seven Seas Marine Mix" (Utility Chemical Company).

The effects of a gradual rise of salinity were examined by gradually adding concentrated sea water to a small quantity of normal sea water containing the experimental animals. Aeration and mixing were effected by bubbling from airstones. Periodically the salinity of the bath was determined with a Goldberg refractometer (American Optical), and samples of the limpet population were removed and placed in running sea water to check for recovery.

Rate of equilibration of limpets to 500% sea water was determined by suspending four specimens of *A. digitalis* by a fine wire from the pan hook of an analytic balance. A beaker of the hyperosmotic solution was raised to cover the limpets, and their weight while immersed was recorded at intervals for 24 hours. Weight while immersed is equal to limpet volume multiplied by (limpet density—solution density), where solution density may be considered constant. Either osmotic loss of water from the limpets, which decreases limpet volume and increases limpet density, or osmotic uptake of salts by the limpets, which increases limpet density, will cause an increase in weight while immersed. Thus this technique permits measurement of osmotic equilibration occurring through either salt or water movements, or both.

The degree of volume regulation, or of osmotic dehydration, was checked by immersing *Acmaea digitalis* on plastic sheets overnight in 400% sea water and comparing loss in wet blotted weight with initial total water (initial wet blotted weight minus dry weight with shell).

Fresh water dilution or evaporative concentration of limpets in the field was measured by drawing 1 microliter samples of E.C.W. into capillary micropipettes from limpets freshly removed from the rock surface. The samples were stored in the capillaries under oil until their concentration was determined with the Chloridometer.

RESULTS

Air, rock surface, and limpet temperatures fluctuated similarly in both Zones I and II. Nighttime temperatures reached a minimum between 03:00 and 06:00. On a few occasions, mild frosts (−2° C) occurred during the winter, but limpet temperatures never dropped below freezing. In the morning, temperatures rose until the sun was past its zenith, then fell gradually until the returning tide caused an abrupt drop to sea surface temperature. The highest temperatures of rock surfaces and individual limpets were recorded on clear, calm, sunny days (Table I) when the lower low tide occurred during the late morning and left much of the intertidal exposed during the hottest part of the day.

The temperatures of limpets and the rock surfaces adjacent to their roosting

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TABLE I

Climatic conditions and maximum temperatures of air, rock surfaces, and limpets on Bodega head

Date (13:00–15:00 Pacific Standard Time)	Weather	Air, °C		Rock surface °C	*A. scabra* °C	Wind, m/sec	
		2 m	1 cm			2 m	1 cm
26 Sept. 70	Sunny, calm, hot	22.5	26	37	37	1.8	0.5
31 May 70	Sunny, calm, hot	22.5	—	38	38	1.8	0.5
Undated, 1968	Sunny, calm, hot	—	—	41	41	—	—
5 Aug. 70	Clear, cool, windy	16	20	27	28	2.7–3.6	1.4–1.8
13 Aug. 70	Intermittent fog, cool, windy						
	Zone I	15	21	26	26	1.8–2.3	0.9
	Zone II	15	22	26	25	1.8–2.3	0.9

sites depended primarily on their orientation to the sun, and to wind and spray. Intertidal height was of secondary importance, serving principally to determine the length of exposure to high temperatures. Since Zone II is exposed for shorter periods, the probability of tidal exposure coinciding with the hottest part of the day is lower than in Zone I, and maximum temperatures usually were lower in Zone II. However, on occasions when both Zone I and Zone II were exposed during the heat of the day, maximum temperatures of sites similarly exposed to the sun could be virtually identical in both zones (Table I).

Nevertheless, in a given area containing all five species, the maximum temperatures of those individual limpets most exposed to solar radiation showed pronounced interspecific differences. Maximum temperatures of *A. scabra* were higher than those of the most exposed *A. digitalis,* which in turn were higher than those of *A. persona, A. pelta* and *A. t. scutum* in the same area (Table II).

TABLE II

Field temperatures of individual limpets most exposed to sun, °C.

	Max.	Mean	Min.	(n)
25 March 1969 15:00				
A. digitalis	30	30	26	10
A. scabra	37	32	28	9
18 May 1971 14:00–15:00				
A. digitalis	29.5	28	26	5
A. scabra	34	34	33	3
A. persona	20	20	19	5
A. pelta	27	23	19	14
A. scutum	27	24	20	11

Table III

Thermal tolerances while immersed. Boldface denotes thermal lethal limits; sample n = *6*

Test temperature °C	% Mortality				
	A. pelta	*A. scutum*	*A. persona*	*A. digitalis*	*A. scabra*
33–34	0				
34–35	**50**	0	0		
35–36	100	**100**	20		
36–37	100	100	20		
37–38	100	100	**80**	0	0
38–39			100	**83**	**17**
39–40			100	100	0
40–41				100	**67**
41–42				100	100

Thermal tolerances of the five species, determined during immersion in sea water, are lower for the Zone II species, intermediate for *A. persona*, and higher for the remaining Zone I species (Table III). Tolerances of *A. scabra* and *A. digitalis*, which experience the widest range of microclimates, were essentially constant regardless of season or collecting site: those of winter-collected samples were at most 1° C below those of summer samples; those of *A. scabra* and *A. digitalis* collected in Zone II were at most 1° C below those of Zone I samples. Thus, there appears to be little acclimation of thermal tolerances.

Thermal tolerances determined under simulated natural conditions were about 5° C higher than tolerances determined during immersion (Table III) for *A. digitalis, A. pelta,* and *A. t. scutum,* and presumably for the remaining species as well. Thermal tolerance seems to be independent of size; no differences were evident between small (9.0 ± 0.27 mm) and large specimens of (16.4 ± 0.28 mm) *A. digitalis*. However, removal of 20% to 60% of the total body water by desiccation did reduce the thermal tolerance of *A. digitalis* by about 3° C.

Both the usual and extreme values of the factors pertinent to desiccation—temperature, humidity (as vapor pressure deficit), and windspeed—are summarized in Table IV. Figures for usual conditions are estimates from measure-

Table IV

Typical and extreme environmental conditions

Weather type	Windspeed m/sec.		Temperature, °C			Vapor pressure deficit mm Hg	
	2 m above surface	2 cm above surface	1 m above surface	1 cm above surface	Surface	1 m above surface	2 cm above surface
Foggy	0–2.2	0–1	8.5–15	15–21	15–27	1–2	1–4
Clear, cold, windy	4.5–13.5	2.2–4.5	6.5–10		15–27	4–5	
Clear, warm, windless (extreme)	0–2.2	0–1	21	27–33	38–41	12–15	23–30

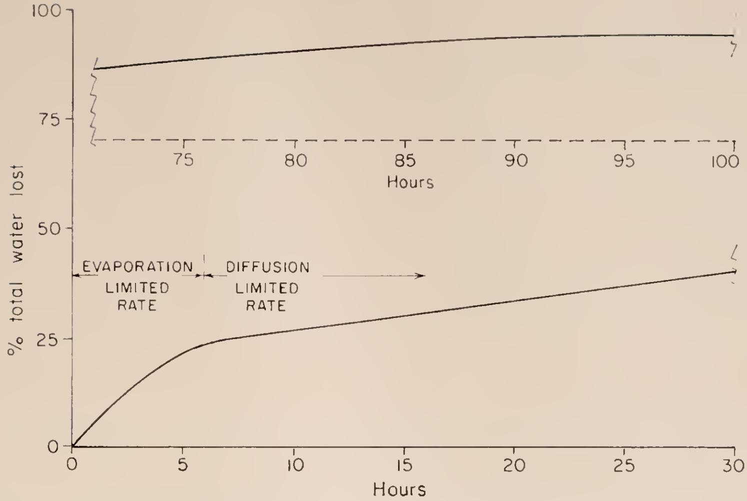

FIGURE 2. Desiccation of permeable bodies under constant conditions: typical time course.

ments taken on "typical" days, while extremes are maximum measurements obtained during several periods of hot weather occurring in 1968–1971. These data were used to determine realistic conditions for desiccation rate and tolerance experiments in the laboratory, and in interpretation of the results.

Desiccation rates of all five species of limpets, living or dead, or limpet shells filled with 15% gelatin, or even small glass vials filled with 15% gelatin, showed a pattern similar to Figure 2 under constant conditions. A high initial rate of water loss, which after a time declines to a lower, fairly constant rate, is characteristic of water-containing permeable bodies exposed to constant drying conditions. The initial rapid loss occurs during the period when evaporation of free water from the surface is the rate-limiting factor; for a given surface this rate is a function of environmental conditions. The subsequent lower rate of water loss represents that portion of desiccation in which free water has disappeared and the rate-limiting factor is the supply of water to the evaporating surface from the underlying matrix. This rate depends on internal diffusion coefficients, which are characteristic of the permeable body. The initial rapid loss rates were highly variable within species of limpets; hence, the diffusion limited portion of the curve, being considered more characteristic of the animals, was used for all comparisons.

All of the species showed a lifting of the shell at high temperatures, cited by Segal and Dehnel (1962) as a mechanism for evaporative cooling. However, this occurred only in water or near-saturated air and was abolished by application of even mild drying conditions. Under desiccating conditions the limpets tend to conserve water rather than using it to regulate body temperature; the shell-lifting response is probably evidence of impending heat coma.

Average desiccation rates under conditions approximating a cool windy day at Bodega Head, using 25–30 individuals of each species, were markedly lower in the Zone I limpets than in the Zone II species (Table V). *Acmaea scabra* was

TABLE V

Desiccation rates under constant conditions; % total water lost/hr; mean ±S.E. ($n = 25$–30); at 18°C: Zone I rates < Zone II rates, $P < 0.05$; differences within zones not significant ($P > 0.05$); at 30°C: A. pelta *rate <* A. t. scutum *rate, $P < 0.05$; all other differences, $P < 0.001$*

	1.4 m/sec 7 mm Hg VPD* 18°C	1.4 m/sec 30 mm Hg VPD* 30°C	2.8 m/sec 30 mm Hg VPD* 30°C
A. digitalis	0.32 ± 0.02	0.66 ± 0.05	1.02 ± 0.11
A. persona	0.28 ± 0.02	3.26 ± 0.18	—
A. pelta	1.02 ± 0.14	5.56 ± 0.28	—
A. t. scutum	1.29 ± 0.10	7.26 ± 0.68	—

* VPD = vapor pressure deficit.

omitted since the serrated shell margins of this species would not fit closely to the plastic discs, as they would to the rock.

Under more rigorous conditions, simulating those occurring during unusually warm weather, all four species tested lost water at higher rates. The increase of rate in the Zone II species was roughly proportional to the increase in vapor pressure deficit, as expected from the general evaporation formula:

$$\text{Evaporation} = \text{K (vapor pressure deficit) C}\left(\frac{\text{windspeed}}{\text{length of evaporating surface}}\right)^{n}$$

(Leighly, 1937). However, the rate increase in *A. digitalis* was only half of what would be predicted on the basis of the formula, implying the existence of some sort of regulation of water loss, as demonstrated by Machin (1965) in his elegant series of papers on desiccation of *Helix*. The increase in desiccation rate of *A. persona* is nearly three times that predicted by the evaporation formula, possibly implying the breakdown of a regulatory mechanism at an elevated temperature not usually experienced by this shade-dwelling limpet. Doubling the windspeed increased the desiccation rate of *A. digitalis* by a factor of 1.5, suggesting that the exponent "n" in Leighly's formula is about 0.5, *i.e.,* that desiccation rate of limpets is roughly proportional to the square root of windspeed (Table V). Desiccation continues even when conditions during exposure do not appear at all stressful; 20 specimens of *Acmaea digitalis,* pre-dried until 20 to 75% total water had been lost, and then exposed outside overnight in fog, continued to lose water (a maximum of 5% total water lost overnight).

Two characteristics of limpets appeared at the outset particularly likely to affect desiccation rates: size and shell circumference. During the course of the study a third factor became evident: an ability of the Zone I limpets to form a mucus sheet between the shell margin and the rock surface.

Intraspecifically, no clear relationship was evident between size and desiccation rates in the laboratory. However, increased body size apparently does result in reduced desiccation rate and mortality in the field, as is indicated by the extracorporeal water concentrations and mortality in three size classes of *A. scabra* collected late in the afternoon after a full day of exposure to warm dry weather

TABLE VI

Chloride concentration in extra-corporeal water and survival of field-desiccated Acmaea scabra

Size group mm	Chloride concentration of E.C.W. (% sea water)		Survival	
	$\bar{x}$ ± S.E.	n_1*	%	n_2*
7–12.9	438 ± 23.2	13	31	29
13–16	356 ± 22.1	10	55	11
17–24	303 ± 22.2	18	63	19

* Difference between n_1 and n_2 is number of individuals from which no fluid was obtainable (internal concentration presumed above 500% sea water).

(Table VI). Interspecifically, the relationships expected on the basis of surface: volume ratios were not observed; the larger species, *Acmaea pelta* and *A. t. scutum,* lost water at higher rates than did the smaller *A. digitalis* and *A. scabra* (Table V).

Since virtually all evaporative water loss occurs through the gap between the shell and the substrate, the relative circumferences in several sets of shells of equal volumes were measured. *Acmaea scabra* was omitted due to the difficulty of estimating circumference and volume, caused by the serrated shell margin. Flat-shelled *A. t. scutum* had the greatest relative circumference, which was at most 25% greater than that of *A. digitalis,* a minor difference compared to the observed differences in desiccation rates.

The third factor of possible significance in determining desiccation rates, the ability of *A. digitalis, A. persona,* and to a lesser extent, *A. scabra,* to form a sheet of mucus between the margin of the shell and the substrate (Fig. 3), was noticed during preliminary desiccation experiments and was subsequently found to be a regular occurrence. The sheet is produced by extension of the mantle until it contacts the substrate, secretion of a layer of viscid mucus on the entire exposed

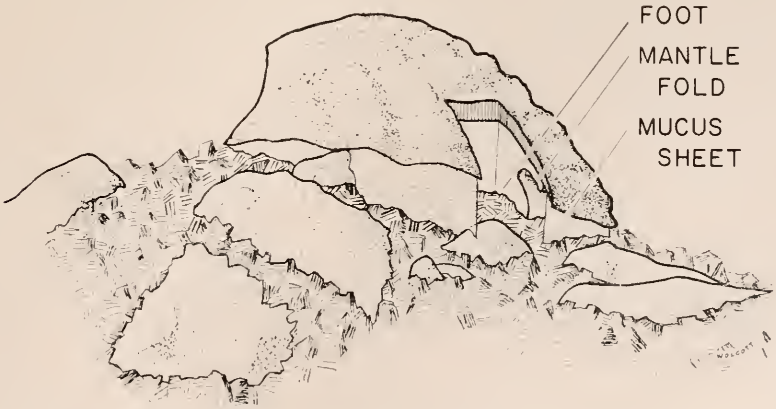

FIGURE 3. *Acmaea digitalis* on rough granite, with mucous diffusion barrier spanning gaps between the shell margin and the uneven substrate.

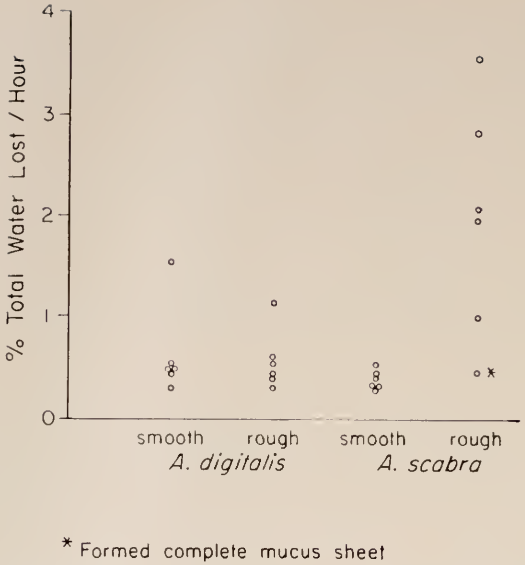

FIGURE 4. Desiccation rates: effect of shell fit to substrate. "Smooth" shells fit substrate closely; "rough" shells fit substrate poorly.

area of the mantle, and subsequent withdrawal of the mantle. The mucus remains in place and gradually dries as the animal within desiccates. Individuals of *A. digitalis* and *A. persona* regularly spanned gaps of up to 15% of the shell diameter; *A. scabra* apparently has a much more limited ability to produce the mucus sheet and usually could not span gaps of this magnitude without leaving perforations in the sheet.

To assess the importance of this mucus sheet in slowing desiccation, desiccation rates of *A. digitalis* with the normal smooth shell margins, *A. digitalis* with chipped shell margins, *A. scabra* with smooth shell margins from the artificial tide system, and *A. scabra* with the normal rough shell margins were compared. All of these animals were desiccated on smooth mylar discs; those with smooth margins fitted the substrate well, while those with chipped or rough margins had gaps between shell and substrate. The smooth-margin *A. digitalis,* rough-margin *A. digitalis,* and smooth-margin *A. scabra* all exhibited about the same rate of water-loss, while the rough-margin *A. scabra* had an average desiccation rate approximately five times higher (Fig. 4). An exception to this was the individual noted by an asterisk, which was the only rough-margin *A. scabra* observed to form a complete mucus sheet. This individual showed a desiccation rate similar to those of the other groups. These results indicate that the normal desiccation rate of *A. scabra* on its homesite is about the same as that of *A. digitalis* which does not home.

The effect of removing the mucus sheet was investigated with four groups of *A. digitalis.* Two of these had the normal smooth shell margins and fitted the Mylar discs well; the other two groups had the shell margins chipped and consequently fitted the discs poorly. Progress of desiccation was followed by periodic

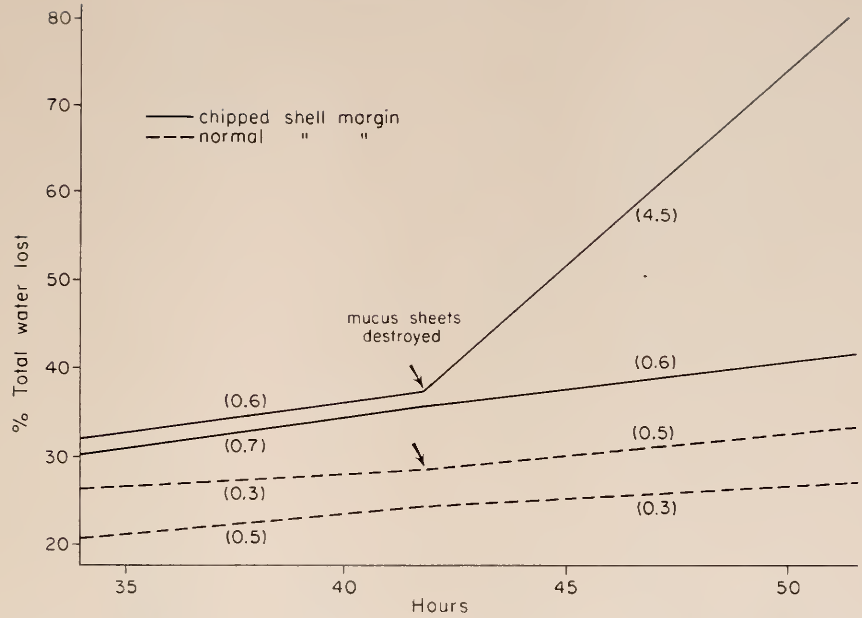

FIGURE 5. Desiccation rate: effect of removing mucus sheet (*Acmaea digitalis*). Lines drawn through points not on graph; 30° C; 30 mm Hg vapor pressure deficit; 1.0 m/sec air flow. Slopes indicated as % total water lost/hour.

weighings, and the mucus sheets of one smooth and one rough group were destroyed after every other weighing. *Acmaea digitalis* regularly repaired the mucus sheet until over 35% of their total water had been lost. Permanently obliterating

TABLE VII

Desiccation tolerances (number surviving/number in interval); 20° C; windspeed 1.0–1.4 m/sec. Boldface denotes interval of 50% mortality (desiccation lethal limit)

% water lost	*A. digitalis*	*A. scabra*	*A. persona*	*A. pelta*	*A. scutum*	Equivalent osmotic concentration (% sea water)
60–62	2/2	2/2	0/0	0/0	3/3	250–263
62–64	1/1	3/1	2/2	0/0	2/3	263–278
64–66	0/0	2/2	1/1	1/1	**2/4**	278–294
66–68	1/1	3/3	1/1	0/0	**2/5**	294–312
68–70	1/1	3/3	2/3	2/2	**4/7**	312–333
70–72	0/0	2/2	0/1	0/0	**3/7**	333–357
72–74	2/2	2/3	2/2	**3/5**	1/3	357–385
74–76	1/1	3/3	2/2	0/0	1/6	385–416
76–78	0/0	1/1	2/2	0/2	0/1	416–455
78–80	0/0	**0/1**	**0/1**	0/3	0/4	455–500
80–82	**3/5**	1/3	1/4	0/2	0/1	500–555
82–84	0/1	0/0	1/3	0/1	0/0	555–625
84–86	0/2	0/1	1/2	0/1	0/0	625–715
86–88	0/5	0/5	0/2	0/0	0/0	715–833

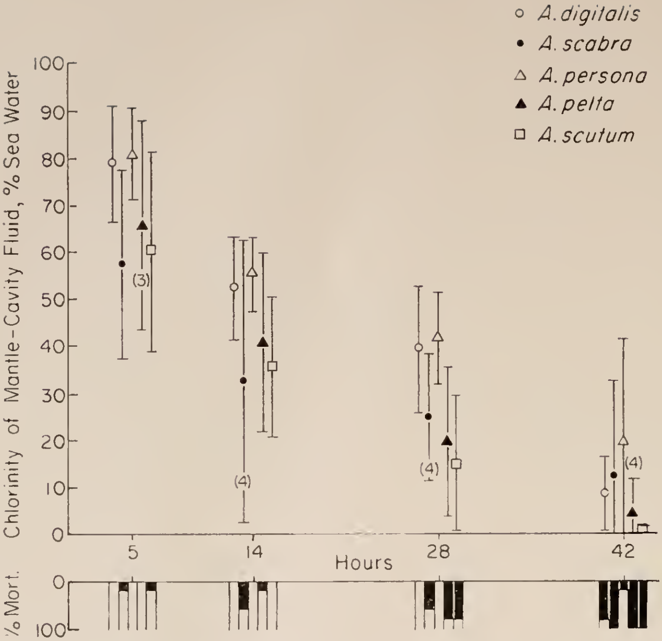

FIGURE 6. Dilution and mortality of limpets during immersion in fresh water. Sample size equals 5 unless otherwise indicated by numbers in parentheses.

the mucus sheet of a representative rough-margin individual of *A. digitalis* caused a seven-fold increase in desiccation rate, while the rates of a representative smooth-margin individual (which repaired the sheet), and of undisturbed rough- and smooth-margin individuals, remained essentially unchanged (Fig. 5).

Desiccation tolerances of the five species of limpets under conditions approximating a cool, breezy day (20° C, 1–1.4 m/sec wind), expressed as per cent of total body water lost and as corresponding internal osmotic concentrations (Table VII), are correlated with the intertidal height of each species' normal habitat.

Acmaea testudinalis scutum, a Zone II limpet, was also desiccated under less severe conditions, which caused it to lose water at a rate similar to that shown by the Zone I limpets under the above conditions. Groups were removed and tested for survival during the second and third days of the experiment. These groups experienced 100% mortality, although many individuals had lost less than 65% of their total body water, indicating that tolerance to desiccation decreases with increasing length of exposure to drying conditions.

To examine the implications of rainwater runoff, all five species of limpets were exposed to standing fresh water for 4, 14, 28, and 42 hours. Chloride concentration of extra-corporeal water (E.C.W.) and survival in each group are plotted in Figure 6. *Acmaea digitalis* and *A. persona* survived somewhat longer than the other species, although no significant differences in equilibration rate are apparent. Longer survival may be the result of slightly greater tolerances to dilution in these

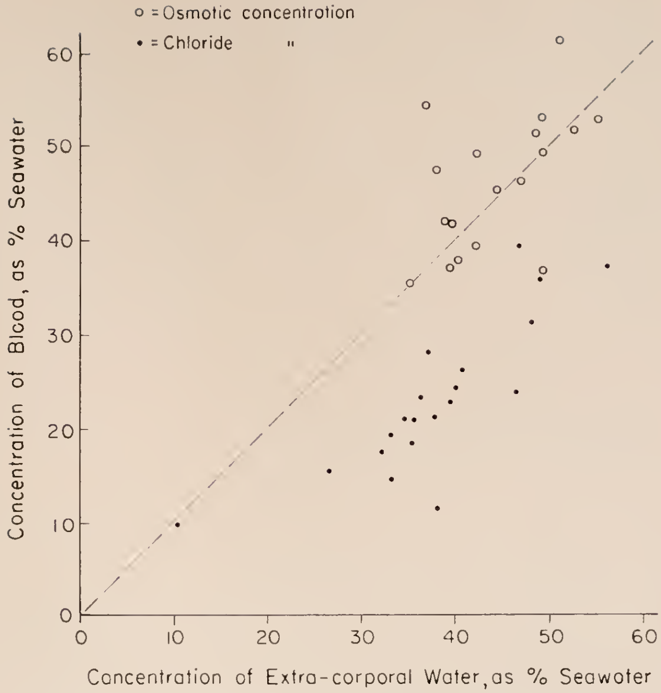

FIGURE 7. Concentration of blood and extra-corporeal water in fresh water stressed *Acmaea digitalis.*

two species. Precise determinations of dilution tolerances were not made since it is impossible to estimate visually when the animals are near death, and excessive numbers of animals would have to be sacrificed to accumulate sufficient data to define the lethal limit. Extra-corporeal water concentrations at which limpets died (Fig. 6) indicate that the limit for all five species is around 30% sea water.

Dilution of E.C.W. caused a decline in blood chloride concentrations to values below those of the E.C.W. However, total osmotic pressure of E.C.W. and blood was found to be the same (Fig. 7), indicating that much of the remaining blood osmotic pressure is due to non-ionic solutes, which osmotically balance the excess salts in the E.C.W.

Fresh-water seepage following rainstorms does leach salts out of limpets in the field. A single tidal exposure in a seep of 1.7% sea water depressed E.C.W. chloride in 5 individuals of *A. digitalis* to 38–40% sea water—near the lethal limit. Presumably extending exposure by another day would have caused death.

The osmotic effects of evaporative water loss were investigated by comparing weight loss and chloride concentration of E.C.W. in laboratory-desiccated animals (Fig. 1). The line represents data calculated for evaporation from an ideal salt solution; the fit of experimental limpet data to this line indicates that salts are being neither voided into the mantle cavity water and urine compartments, nor sequestered in the body during the progress of desiccation. Internal osmotic concentra-

TABLE VIII

Water content of five species of Acmaea, including two size classes of A. scabra

Species	% Water in total hydrated weight of soft parts (mean ± S.E.)
A. digitalis	85.81 ± 0.24 (n = 62)
A. scabra	86.81 ± 0.27 (n = 68)
0.3–0.8 g	87.00 ± 0.38 (n = 9)
1.4–2.4 g	86.90 ± 1.09 (n = 11)
A. persona	85.88 ± 0.29 (n = 24)
A. pelta	88.73 ± 0.32 (n = 36)
A. scutum	87.33 ± 0.48 (n = 25)

A. digitalis
A. pelta > *A. scabra* ($P < 0.05$)
A. persona

A. scutum > *A. digitalis* ($P < 0.05$)

All other differences not significant at 5% level.

tions expected in animals of each species at their respective lethal limits of desiccation (Table VII) were obtained from Figure 1.

Chloride concentrations of E.C.W., where fluid was still obtainable, and survival of three size groups of *A. scabra* collected after a warm dry day are shown in Table VI. Concentrations in the smallest group are significantly ($P < 0.05$) higher than in the largest group, as is the proportion of animals with no obtainable fluid (chloride concentration presumed above 500% sea water). Survival increases with increasing size.

Water content of fully hydrated limpets established on Mylar discs

(Total Water, Hydrated/Weight of Soft Parts, Hydrated)

was similar for all five species and for both large and small *A. scabra* (Table VIII).

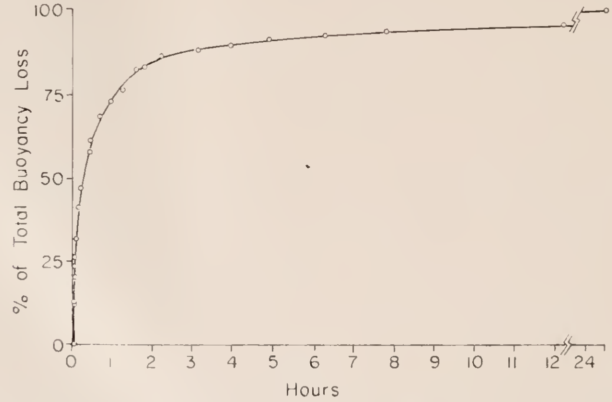

FIGURE 8. Equilibration of *Acmaea digitalis* to 500% sea water, as shown by buoyancy changes.

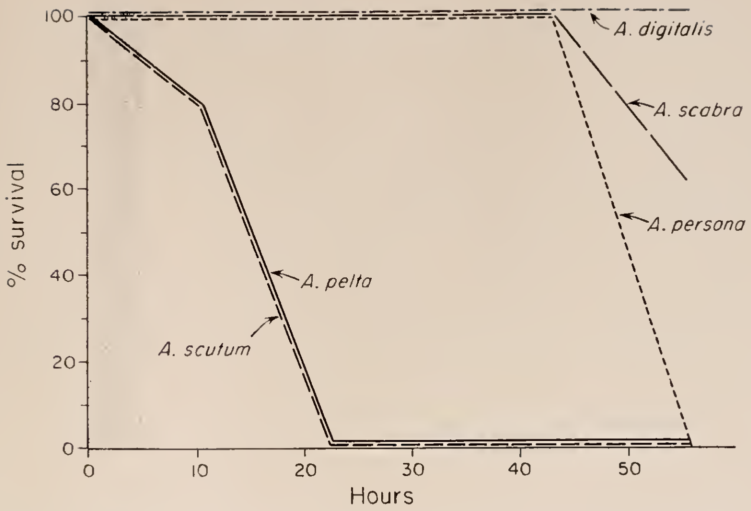

Figure 9. Survival in 400% sea water. Concentration raised from 100% to 400% sea water during first 5 hours (stippled area).

All five species tolerated 5-hour immersion in concentrations up to 400% sea water; at 500 and 600% sea water the majority of *A. pelta* and *A. scutum* perished while the majority of the Zone I limpets survived. Equilibration of *A. digitalis* to 500% sea water, as shown by changes in weight while immersed due to water loss and salt uptake, was 90% complete in 4 hours (Fig. 8).

Under more natural conditions of gradual salinity rise, the Zone I limpets tolerated 400% sea water longer than the other species (Fig. 9). The osmotic stress produced by immersion in 400% sea water is equivalent to that produced by evaporative loss of 75% of the total water. However, since limpets perform limited volume regulation, osmotic dehydration in 400% sea water removes only 28% ± 5% (mean ± S.E.; n = 10 *A. digitalis*) of the total water. Thus the effect of the same osmotic stress produced by prolonged desiccation can be examined in the absence of excessive tissue dehydration.

Gradually raising salinity over 20 hours to 250% sea water simulated the osmotic effect of evaporatively removing 60% of the total water, a level well below the acute tolerances of even *A. pelta* and *A. t. scutum*. Nevertheless, both *A. pelta* and *A. t. scutum* began to die early in the second day of exposure and all were dead within 33 hours.

Discussion

Are range limits of animal populations determined by physiological tolerances to abiotic environmental factors, or does some biotic factor—*e.g.*, behavior or competition—prevent occupation of the entire physiologically acceptable range? The intertidal zone is an obvious habitat in which to explore this question, since it represents one of the sharpest gradients in physical conditions to be found on this planet. The marine grades into the terrestrial environment over the space of a few meters, and animals invading this zone from the sea encounter progressively greater physiological challenges. Obviously, these animals cannot survive above some critical level in the intertidal, determined by the extent of their dependence on sea water. In this sense their ranges are potentially limited by the intolerable conditions prevailing above that level. However, it remains to be demonstrated that they do live everywhere that physical conditions are tolerable, and that partitioning of the intertidal gradient is thus determined principally by interspecific differences in physiological tolerances.

The acmaeid limpets present a convenient system in which to approach this problem, because there are several abundant species which collectively occupy the entire intertidal range. Like the barnacles, they are abundant and easily accessible, but have the additional advantage of being removable, allowing manipulative and laboratory experiments. Adult limpets live several years (Frank, 1965a; Sutherland, 1970) and are thus exposed to the climatic extremes occurring in all seasons. The response of all of these limpets to tidal exposure is immobility—the "clamp-down response" of McAlister and Fisher (1968). The animals are thus behaviorally trapped and must endure any stressful conditions until the tide returns. The aquatic microenvironment of such "roosting" limpets is the small quantity of water retained under the shell. Due to its minute volume, the thermal, evaporative, and osmotic effects of exposure are far more rapid and dramatic than would be the case in organisms protected by the larger water mass of tidepools.

The physical environmental factors representing potential stresses to limpets differ according to season. During the winter, both rainwater runoff during storms and seepage of freshwater from the cliffs present potential osmotic problems. Limpets appear to have no defense, other than the "clamp-down response," against dilution by fresh water. Like most other gastropods, they are osmoconformers. Fluctuations in the osmotic concentration of mantle-cavity fluid from 23% to 150% sea water are closely paralleled by fluctuations in concentration of the three major internal fluid compartments (urine, blood, and intracellular fluid) in *Acmaea limatula* (Segal and Dehnel, 1962), from 50% to 125% sea water in *A. t. scutum* (Webber and Dehnel, 1968). Blood of *A. digitalis* is in osmotic equilibrium with diluted extra-corporeal water (mantle-cavity water and urine); blood chloride levels are even lower than those of the extra-corporeal water, presumably due to the presence of osmotically active organic compounds in the blood (Fig. 7).

Despite their apparent vulnerability to dilution by fresh water trickling down the rock surface, the various species of *Acmaea* show no pronounced differences in either resistance to dilution or tolerance of depressed internal electrolyte concentrations (Fig. 6), suggesting that no strong differential selection for these traits is taking place in the field. This seems to be true in the vast majority of the rocky intertidal at Bodega Head. Rainfall usually comes during storms, which bring high winds and rough seas. The effective tidal level is elevated by the resulting splash and salt spray. Consequently, the actual maximum period of vulnerability to rainwater runoff is shorter than the tidal exposure predicted on the basis of calm sea conditions. It is therefore unlikely to exceed 24 hours in Zone I, and must be less than 19 hours in Zone II (Sutherland, 1970). All of the species tested, with the exception of *A. scabra,* are able to withstand immersion in fresh water for longer than the maximum expected exposure in their natural habitat (Fig. 6). (*A. scabra* was prevented by its very irregular shell margin from effectively clamping down on the flat plastic substratum during immersion in the laboratory, and consequently lost salts at a disproportionately high rate. The close fit of shell to rock would presumably abolish this effect in the field.) Furthermore, since rain at Bodega Head generally falls in a series of brief showers, it is improbable that rain would fall continuously for even 19 hours coinciding with a period of tidal exposure. Thus lethal, or even stressful, dilution by winter rainfall appears to be an extremely unlikely situation for even the highest of the limpets.

An exception occurs in areas which form fresh-water seeps during the winter and spring months. Many of these areas are located in embayments in the cliffs and support a heavy film of algae, representing a rich food resource. Due to their geometry they are kept wet with salt spray when storm surf is running. During winter storms *A. digitalis* normally moves upward, then moves down in summer (Frank, 1965a; Miller, 1968); at Bodega it seems to move upward into the seep areas under the protection of the storm-wave spray. When a storm brings a large amount of rain and is followed by calm weather and low seas, the result is both an augmentation of the seepage rate and a drastic reduction or cessation of salt-spray falling on seep areas. Limpets which have moved into the path of the seeping fresh water risk becoming trapped by their clamp-down response and suffering fatal dilution unless subsequent tides bring sufficient salt-spray to enable them to move out of the area. There may be small interspecific differences in adaptation to these conditions; the slightly longer survival of *A. digitalis* and *A. persona* in fresh water (Fig. 6) is correlated with the observation that only these species move into the seep areas. It is also possible that lethal dilution occurs, limiting further exploitation of seep areas: in a seep area the day after a storm (19 January 1970), extra-corporeal water concentrations of *A. digitalis* were as low as 38% sea water, and subsequently all limpets disappeared from the area. The data are not conclusive but suggest that the penetration of seeps and the extent of their utilization by Zone I limpets are limited by physical factors.

During the spring and summer, virtually no rain falls on Bodega Head, and the potential limiting factors are solar heating and desiccation. Adaptive interspecific differences in temperature tolerance do exist. Thermal lethal limits during immersion are correlated with the microhabitats in which the various species are normally found (Table III), as is the case in many groups of intertidal animals (Gowanloch and Hayes, 1926; Evans, 1948; Gunter, 1957). The highest tolerance is shown by *A. scabra,* which occupies primarily horizontal surfaces in Zone I, often directly exposed to the full force of the sun's rays (Haven, 1970) and which is limited in mobility by its homing habit. *Acmaea digitalis* has a somewhat lower tolerance; it generally occupies more vertical or overhanging surfaces in Zone I (Haven, 1970) which are shaded during much of the day. *Acmaea persona*, the third Zone I species, shows strong negative phototaxis, spending daylight hours chiefly in dark crannies or under boulders; it has a lower tolerance yet. The Zone II limpets, *A. pelta* and *A. t. scutum,* are characteristically found on the walls of shady surge channels or in the shelter of macroscopic algae (*e.g., Porphyra, Pelvetiopsis, Endocladia*) and are seldom exposed long enough to experience extreme solar heating. They have the lowest temperature tolerances of the five species tested.

These thermal tolerances show little or no seasonal acclimation. This, although in contrast to the situation reported for lethal limits of some intertidal molluscs on English shores (Newell, Pye, and Ahsanullah, 1971), is not suprising. Limpets would be expected to show acclimation responses to regular, predictably changing environmental variables, such as sea water temperatures, and not to transient, unpredictable stimuli such as sudden periods of high temperatures during tidal exposure. In England, rising sea water temperatures precede the spring and summer, when thermal stress during exposure is likely; thus they serve as a cue for compensatory acclimation. On the other hand, spring and summer on the California coast are preceded by minum sea water temperatures (Sutherland, 1970). Thus, although other functions in *Acmaea* have been shown to acclimate seasonally (*e.g.,* heart rate; Segal, 1956b), any compensatory acclimation of thermal lethal limits in response to changes in sea water temperature would be non-adaptive. In the absence of acclimation, the lack of intraspecific variation in thermal tolerance among individuals collected from different zones or microhabitats is to be expected. The free spawning habits and pelagic larvae of limpets ensure thorough mixing of gametes from all tidal levels, and preclude the local formation of physiologically different races.

The existence of interspecific differences in thermal tolerance, correlated with distributions, supports the hypothesis that high temperatures serve as a limiting factor in limpet zonation. However, the second criterion necessary to establish the hypothesis is not fulfilled. Rock surface temperatures never exceeded the lethal limits (as determined under simulated natural conditions) of even *A. pelta* and *A. t. scutum,* the most susceptible species tested. The observed differences in physiological adaptations to high temperatures are probably concerned with adjustment of optimal temperature ranges and with coping with extended sublethal thermal stresses, rather than with extension of lethal limits. On the basis of thermal lethal limits alone, any of the species could theoretically live anywhere in the Bodega Head intertidal.

In actuality, none of the species is found throughout the entire intertidal zone; each is found in a characteristic thermal microclimate. The highest limpet temperatures were observed in *A. scabra* which had homesites on rock surfaces perpendicular to the sun's rays. Similar temperatures certainly occurred at similarly exposed sites available to other limpets elsewhere in Zone I, and probably in Zone II. However, diligent search revealed that *A. digitalis, A. persona, A. pelta,* and *A. t. scutum* invariably occupied microsites which were shaded most of the day or received only oblique sunlight—never those directly exposed to the sun. These roosting sites were always several degrees cooler than the more horizontal, sun-baked sites occupied by maximally exposed *A. scabra* (Table II). They were never observed to approach lethal temperatures of the occupants, even assuming lethal limits to have been depressed several degrees by desiccation. Although nonhoming limpets often shift their roosting sites (Frank, 1964), they are never found in "hot spots." This suggests that the observed distribution is due to behavioral selection of "safe" microsites by these species, as in Zone I *Littorina* (Bock and Johnson, 1967), rather than to elimination of less tolerant limpets which wander into exposed locations. *A. scabra* is unique among the species examined, in that many individuals are restricted to the potentially most stressful areas of the intertidal by their homing habit and resultant low mobility.

No temperatures above the lethal limit of *A. scabra* have been observed at Bodega Head, but such temperatures much occur toward Cape San Lucas, Baja California, the southern limit of this species' range (Fritchman, 1961). The fact that *A. scabra* prefers horizontal surfaces (Haven, 1970) and does not avoid sites fully exposed to the sun, suggests that in the southern portion of the geographical range mortality due to extreme environmental temperatures may set the upper intertidal distribution limit of this species. On the other hand, it seems unlikely that heat death will be found to limit intertidal distributions of the other species even at the southern range limits, in view of their ability to select tolerable microhabitats. It seems more likely that they would simply select increasingly protected roosting sites at lower latitudes. Geographical range limits in these species may be the result of reproductive failure, either in adult gametogenesis or larval survival, as concluded by Fritchman (1961) on the basis of gonad index cycles. Testing of this hypothesis must await the development of culturing techniques for adults, larvae, and settling stages of *Acmaea*, which has only begun (Kessel, 1964).

From the foregoing it is clear that high temperatures do not of themselves limit the intertidal distributions of *Acmaea* on Bodega Head. Nevertheless, several data implicate high temperatures as an important contributing factor. Exceptionally warm weather was associated with all of the limpet "kills" previously reported (Orton, 1933; Hodgkin, 1959; Frank, 1965a; Sutherland, 1970), and with most of those observed during the course of this study. Although small *Acmaea* do not have lower lethal limits than their larger brethren, Davies (1970) has reported that the small individuals of *Patella* reach the highest temperatures in the field. It is the small *Acmaea* that seems most susceptible during "kills" (Table VI).

One of the physical effects of increasing temperature is an increase in the vapor pressure of fluids, and hence in evaporation rates. Desiccation rate of *Patella* under laboratory conditions increases with decreased body size, and the same relationship seems to hold for *Acmaea* in the field (Table VI). In all cases of "kills," deaths were observed in the high intertidal after two or more days of warm weather. A hypothesis consistent with all of these data is that cumulative, eventually lethal desiccation is the "limiting factor" determining the partitioning of the intertidal by these five species of *Acmaea*. If this hypothesis is correct, again two criteria should be satisfied. First, the limpets should show interspecific differences in adaptation to drying conditions, permitting them to exist in microhabitats exhibiting differing degrees of desiccation potential. Secondly, desiccation should be found to at least occasionally cause mortality in the field, thus restricting ranges at different levels depending on the physiological tolerances. The first criterion is satisfied, since differences exist in both desiccation rates and desiccation tolerances. However, the second criterion is satisfied in only some of the species on Bodega Head.

Desiccation rates of *A. pelta* and *A. t. scutum* are significantly higher than those of the Zone I species under mildly drying conditions (Table V). A similar pattern was found among British limpets by Davies (1969); high-shore *Patella* *vulgata* show lower desiccation rates than low-shore *P. vulgata* or *P. aspera.* Unfortunately actual rates cannot be compared between the two studies because Davies exposed his limpets to dry, still air in desiccators, whereas moving air was used in the present study. Increasing the severity of drying conditions causes a proportionately greater increase in the rates of the Zone II *Acmaea;* under conditions common in Zone I on warm days, Zone II limpets may desiccate up to 11 times faster than Zone I limpets (Table V, column 2).

Three characteristics of the limpets were examined as possible mechanisms of the interspecific differences in desiccation rates: body size, shell shape, and the production of a mucus sheet between shell margin and rock. Size does appear to be an important factor in intraspecific variation in desiccation rate. Small specimens of *A. digitalis* are less tolerant of prolonged drying than are larger individuals (Frank, 1965a) and smaller individuals of *Patella vulgata* and *P. aspera* have higher rates (integrated evaporation-limited and diffusion-limited) of water loss (Davies, 1969). Desiccation of limpets under constant conditions on Mylar discs showed no clear relationship between size and diffusion-limited desiccation rate, although small animals apparently do desiccate faster in the field (Table VI). These data suggest that the size effect is most pronounced during the early evaporation-limited (Fig. 2) drying of the surface and immediately underlying tissues. In smaller limpets this involves a larger proportion of the total water; consequently, its more rapid loss gives desiccation a "head start" and leads to earlier mortality under prolonged exposure.

However, size differences and the attendant surface-volume relationships do not account for the observed interspecific differences in desiccation rates. *Acmaea pelta* and *A. persona* fall into roughly the same size range, yet have widely disparate desiccation rates. On the other hand, *A. digitalis* and *A. scabra* are smaller and thus have higher surface-to-volume ratios than *A. pelta* or *A. t. scutum*, yet have much lower rates.

Shell shape is not the determining factor either. *Acmaea testudinalis scutum* is considerably flatter than the other species, thus having a greater relative circumference, hence a longer aperture through which water vapor may escape, and shows the highest desiccation rates (Table V). However, *Acmaea persona* and *A. pelta* have virtually identical relative circumferences, yet *A. pelta* loses water much more rapidly. Furthermore, even the greatest differences in shell circumference (about 25%) are insufficient to account for the differences in desiccation rate observed between Zone I and Zone II limpets.

The ability to form a mucus sheet between shell margin and substrate (Fig. 3), which occurs in the species with low desiccation rates, is by far the most important adaptation. Removal of an intact sheet increases the desiccation rate by approximately seven fold (Fig. 5), causing *A. digitalis* without its mucus sheet to lose water at approximately the same rate as would *A. pelta* under the same conditions (Table V). It appears that this single mechanism accounts for almost the entire difference between desiccation rates of Zone I and Zone II limpets. It is further indirect evidence of the adaptive value of the mucus sheet that the limpets will repair one that has been damaged or obliterated, despite the short-term sacrifice of considerable water. *Acmaea digitalis* replaced completely destroyed mucus sheets up to five times, becoming incapable of doing so when more than 35% of the total water had been lost.

The mucus sheet lowers the desiccation rate of *A. digitalis,* which does not fit the rock, to the same level shown by *A. scabra* having an almost perfect fit of shell to rock (Table V; Fig. 5). Thus the effect of "homing" is not to give *A. scabra* a much lower desiccation rate than possessed by any other species, as has been suggested by Haven (1970). Thus the function, as well as the mechanism, of this complex behavioral phenomenon have yet to be adequately explained, in spite of copious research (Russell, 1907; Pieron, 1909; Wells, 1917; Orton, 1929; Abe, 1940; Hewatt, 1940; Villee and Groody, 1940; Edelstam and Palmer, 1950; Thorpe, 1963 (review); Funke, 1964; Galbraith, 1965; Cook, Bamford, Freeman and Teideman, 1969; Thorne, 1969). The homing habit restricts *A. scabra* to the area within which it can forage and return to its homesite during a single tidal immersion. In contrast, *A. digitalis* and *A. persona* do not require a homesite for minimal desiccation rates, and *A. digitalis* is known to lead a more opportunistic existence, moving with seasonal changes in food and exposure (Frank, 1965a). If the reduction in mobility required by homing is a disadvantage, as seems logical, it presumably must be balanced by some unique advantage. Many *A. digitalis* which by virtue of their mucus sheets already have low desiccation rates, and a few *A. pelta,* which do not, have been observed to home on Bodega Head. This suggests that the advantage of homing may be primarily related not to desiccation but to some other environmental factor, *e.g.,* dislodgement by wave impact or injury by water-borne sand swept under the shell margin.

Adaptive differences are also evident in the desiccation tolerances of the five species. Under mild desiccating conditions the Zone I limpets will tolerate loss of about 80% of their total water, while the Zone II limpets will tolerate loss of about 70%. Evaporative loss of such a large proportion of the total water must have profound osmotic implications, unless the limpets have some means of maintaining their internal concentration below that of the extra-corporeal water as it becomes concentrated by evaporation. Regulation of blood concentration does not occur; equilibration with solutions of high salinity is rapid (Fig. 8). An ability to displace some excess electrolytes from the cells, by accumulating amino acids or other organic solutes during hyperosmotic stress, exists in some molluscs (*e.g., Tegula funebralis;* Peterson and Duerr, 1969). Although in this study no analyses of the intracellular fluid compartment were made, the absence of excess chloride in the urine (Fig. 1) over that predicted for evaporation from an ideal salt solution suggests that in *Acmaea* no such mechanism exists. This implies that concentration of the extra-corporeal water must result in a similar increase in electrolyte concentration of the blood and even of the intracellular fluid.

If it is the osmotic effects of desiccation which are crucial, then the apparently minor differences in desiccation tolerances—from about 70% total water lost in the Zone II species to about 80% in the Zone I species—are not so minor. The osmotic concentration of body fluids resulting from desiccation is not proportional to the per cent water lost, but to the inverse of the per cent water remaining. Hence, the internal osmotic concentrations corresponding to the lethal desiccation limits (Table VII) range from about 280–380% sea water for Zone II limpets to 450–550% sea water for the Zone I limpets—a substantial difference indeed.

The osmotic concentrations corresponding to desiccation tolerances are closely paralleled by the osmotic tolerances determined by 5-hour immersion in hyperosmotic solutions: Zone II limpets tolerate up to 400% sea water; a majority of Zone I limpets survive up to 600% sea water. The slightly higher tolerances during immersion are presumably due to the shorter exposure to osmotic stress—5 hours during immersion *vs.* 1–8 days during the course of desiccation.

All of these limpets are remarkably tolerant of both desiccation and osmotic extremes. Their desiccation tolerances are comparable to those of some chitons (75% total water lost; Boyle, 1969), and are higher than those reported by Davies (1969) for *Patella* (30–65%). The desiccation tolerances of *Acmaea* are among the highest recorded for any animals, including desert amphibians (60% total water lost in *Scaphiopus;* Thorson and Svihla, 1943) and desert insects (55% total water lost in tsetse flies; Bursell, 1959). The ability of larval chironomids (*Polypedilum vanderplanki* from African desert rockpools) to survive complete desiccation (Hinton, 1950) of course represents the ultimate in adaptation along this line, but renders the tolerances of the essentially marine limpets only slightly less impressive. The range of external, and hence internal and intracellular concentrations tolerated by all five species—30% to 400% sea water—is also extremely wide when compared with published tolerances of other euryhaline molluscs. Among the hardiest are the intertidal estuarine mussel *Modiolus* (8.5–137% sea water; Pierce, 1970); the intertidal pulmonate *Siphonaria* (31–230% sea water; Allanson, 1958); the intertidal chiton *Sypharochiton* (50–150% sea water; Boyle, 1969); and the ventricle-strip preparation from the mussel *Mytilus* (10–200% sea water; Pilgrim, 1953). It must be borne in mind, however, that most of these studies of osmotic tolerances involve extended exposures (*e.g.,* 60 days; Pierce, 1970); the administration of fresh water or 400–600% sea water for such lengthy periods would represent a highly unnatural situation. The possibility of extreme osmotic fluctuations occurring during relatively short periods, as might happen in exposed animals during tidal emersion, appears to have been overlooked. Thus, with the exception of data for *Sypharochiton* (tolerates 75% total water lost, equivalent to 400% sea water; Boyle, 1969) and *Siphonaria* (tolerates 225% sea water for short periods; Allanson, 1958), few strictly comparable measurements of osmotic tolerances are available for other molluscs.

In addition to the differences in absolute tolerances of desiccation and its osmotic effects, there are differences in the duration of stressful conditions that the limpets will survive. *Acmaea digitalis, A. scabra,* and *A. persona* under mildly drying conditions (18° C, 68% R.H., 1.4 m/sec airflow) survived up to eight days. On the other hand, *A. scutum* experienced 100% mortality by the second day of exposure to even less severe conditions, before high levels of desiccation had been reached. Apparently the Zone II species will not tolerate drying conditions for periods much in excess of 24 hours. Similarly, Zone I limpets will tolerate 400% sea water for several days, whereas Zone II limpets succumb within 22 hours (Fig. 9).

The ability of the Zone I limpets, especially those of small size, to tolerate desiccation and its osmotic effects for such extended periods has been attributed to stores of extra water held under the shell. The relative water-holding capacity of the shell is larger in smaller limpets (Abe, 1931; Segal, 1956a); and water-holding capacity or "extra-visceral space" is larger in high intertidal species of *Acmaea,* or high-intertidal subsamples within species (Shotwell, 1950a; Segal, 1956a). Segal and Dehnel (1962) found that removing the "extra-visceral water" (mantle-cavity water and voided urine, more accurately "extra-corporeal water") from *A. limatula* before subjecting the limpets to desiccation in the field slightly accelerated concentration of body fluids. However, the maximum difference between concentrations in the experimental animals and the controls was only about 13%, and had virtually disappeared after four hours, when the blood concentrations of both groups had not risen beyond 120% sea water. Segal and Dehnel concluded from these data that the extra-corporeal water performs a significant function in retarding desiccation. However, the transient delay in concentration of body fluids appears to be a small difference compared with the duration of desiccation and the levels of osmotic concentration which are likely to be stressful. Segal and Dehnel's data show that *A. limatula* easily tolerates 150% sea water for 48 hours, and all five species of *Acmaea* in this study would tolerate 400% sea water for the entire duration of Segal and Dehnel's desiccation experiment.

Segal (1956a) and Segal and Dehnel (1962) refer to the extra-corporeal water as though the limpets could expend this store before they were obliged to sacrifice body water; in such a compartmentalized situation the augmentation of the extra-corporeal water might serve an important function in delaying concentration of body fluids. However, it has been shown that there is no osmotic barrier between the extra-corporeal water and body fluids (Fig. 7; Segal and Dehnel, 1962; Webber and Dehnel, 1968). Therefore, it is not the amount of water stored in the "extra-visceral space," but the total amount of water stored under the shell, including body water, that is the significant measure of osmotic buffering ability. Increases in water-holding capacity are significant only if they result in increased water-holding. This is not the case in the Zone I species of *Acmaea*, which actually retain a few per cent *less* water than *A. pelta* and *A. t. scutum* (Table VIII). Neither do the small limpets hold relatively more water than the large ones (Table VIII). Observations show that, shell capacity notwithstanding, limpets expel or resorb most of the extra-corporeal water within the first few hours of desiccation under laboratory conditions. It therefore seems that "adaptive differences" in water-holding capacity are a laboratory artifact and are unimportant in delaying the desiccation of small or high-intertidal limpets. The observed differences in desiccation tolerance must therefore involve not morphological differences, but differences in physiological adaptation to some potentially lethal effect of desiccation.

There is a striking parallel between desiccation tolerances and osmotic tolerances. However, the question remains whether evaporative concentration of body fluids is what leads to death during desiccation, or whether some other effect of desiccation supervenes. Accumulation of toxic anaerobic metabolites during exposure, as may occur in the barnacle *Chthamalus* (Barnes and Barnes, 1964), is unlikely in *Acmaea* since the limpet continues to take up oxygen via the mantle fold during exposure (Baldwin, 1968; Kingston, 1968). Work on other intertidal molluscs suggests that such aerial respiration is common; it may be lower (Helm and Trueman, 1967; Lent, 1968; Paine, 1971) or higher than respiration while submerged (Micallef and Bannister, 1967, Sandison, 1968) but is reduced as desiccation progresses (Sandison, 1966). Irreversible damage to the respiratory surfaces by drying does not appear to be the direct cause of death, since limpets observed to have dried, stiff integuments often survived. Nor does lack of water as a biological solvent appear to be the cause of mortality as is implied by McAlister and Fisher (1968) for the false limpet *Siphonaria pectinata*. Immersion of limpets in hyperosmotic solutions causes much less dehydration than does desiccation to the same internal concentration. Nevertheless, *A. t. scutum* and *A. pelta* die after less than 1.5 days exposure to a solution gradually concentrated to 250% sea water, just as they do if a similar rise in internal concentration is produced by desiccation with all of its attendant effects. The only stress common to the two experimental regimes is high osmotic concentration. No effects of desiccation, other than evaporative concentration, appear to cause death of limpets during drying; on the other hand, effects of osmotic stress associated with desiccation are sufficient to account for all desiccation-induced mortality. It therefore seems clear that the mechanism by which desiccation causes death in *Acmaea* is through concentration of the body fluids to lethal levels. The ability of the Zone I limpets to withstand severe, prolonged desiccation is a result of physiological adaptation to high electrolyte concentrations at the cellular level.

From the foregoing it is evident that interspecific differences in adaptation to drying conditions permit the observed partitioning of the intertidal zone by *Acmaea*. It is by virtue of their high desiccation tolerances, low desiccation rates, and ability to endure being in a desiccated state (or under hyperosmotic stress) for extended periods, that the Zone I limpets are able to exploit the otherwise inaccessible resources of the high intertidal. Their different mechanisms for achieving low desiccation rates dictate different life-styles. *Acmaea scabra* requires a close-fitting "homesite" to prevent rapid water loss; its foraging range is therefore limited to the area within which it can feed and return during a single tidal submersion—about 6 hours at best (Sutherland, 1970). *A. digitalis,* due to its ability to form the mucous diffusion barrier, is not dependent on a close fit to the rock, and is free to lead a more opportunistic existence. It seems to be able to outcompete *A. scabra* in most of Zone I (Haven, 1973), except in the most exposed, sun-baked sites. *A. scabra*, unlike *A. digitalis,* does not avoid these areas and, in part due to its adaptation to high temperatures, is able to exclusively exploit them. *A. persona* uses a third strategy, moving and feeding during the hours of darkness and hiding in crevices during the day; presumably this reduces competition with the other Zone I species in addition to lowering desiccation rates.

The Zone II limpets, however, do not have the physiological adaptations necessary for existence in Zone I. They lose water many times as fast as would Zone I limpets in similar circumstances (Table V); in addition, they have lower tolerances to desiccation and the resultant high internal osmotic concentrations (Table VII). The result is that, under conditions common in Zone I (Table V, column 2) they will reach their lethal limits in 6–10 hours. Furthermore, they are incapable of surviving in a desiccated state, or in a correspondingly concentrated salt solution, for more than 20–24 hours (Fig. 9). Thus, although this degree of adaptation allows *A. pelta* and *A. t. scutum* to survive in Zone II where exposure never exceeds 19 hours (Sutherland, 1970), they cannot survive in Zone I, where exposure may last up to 8 days. The upper range limits of these species may therefore be considered *potentially* limited by desiccation. However, they do not seem to ever attempt to migrate upward until stopped by their physiological limitations. No kills, or even near-lethal degrees of desiccation, have ever been observed in *A. pelta* or *A. t. scutum* in the field. Apparently the upper range limits of these two species are not functionally determined by physical factors causing desiccation, but by behavioral adaptations leading to selection of physiologically tolerable microsites.

On the other hand, desiccation mortality does appear to have important effects on limpet populations in Zone I. Numerous specimens of *A. scabra* and *A. digitalis* have been found dead in the field on several occasions, usually after periods of unusually warm weather. Such kills seems to take a greater toll of small limpets (Table VI), presumably due to the greater surface-to-volume ratio and resultant higher initial rate of water loss. Characteristically, size distributions of limpets and other molluscs in the high intertidal are heavily skewed toward older individuals (Das and Seshappa, 1948; Frank, 1965a; Sutherland, 1970). This may very well be the result of higher desiccation mortality in smaller size classes, as suggested by North (1954) and Boyle (1970).

In each kill the dead limpets were found only at the upper limits of the species' intertidal ranges. The most extensive kill observed during the course of this study (11 August 1971) was preceded, not by heat, but by cool, foggy, almost windless weather. The sea had been exceptionally calm, and areas which normally receive abundant spray had been left dry for at least a week. Hundreds of limpets up to 11 years old (estimated from calculated growth curves in Frank, 1965b, and Sutherland, 1970) were eliminated from the upper reaches of these areas. Similar kills have eliminated limpet populations from high Zone I areas elsewhere on Bodega Head (Sutherland, 1970) as well as on Oregon (Frank, 1965a) and British coasts (Orton, 1933). Kills of *A. persona* have not been observed, possibly due to the scarcity of this species in the study area. However, desiccation-induced mortality obviously does set the upper range limits of *A. digitalis* and *A. scabra* on Bodega Head.

This brings us full circle to the question, "Do physical environmental conditions limit the ranges of animal populations?" Clearly they do in some cases. The physiological adaptations of *A. digitalis* and *A. scabra* to desiccating conditions permit their exploitation of Zone I, and desiccation mortality sets the upper limit of their ranges. However, it is equally clear that physical environmental conditions do not limit ranges in some other cases. Upper range limits of two otherwise very similar species, *A. pelta* and *A. t. scutum*, are determined by their behavior, not desiccation mortality.

As so often happens, one set of answers has led to another question: "Under what circumstances are physical factors likely to limit the ranges of animal populations?" It seems logical that animals which live in marginal habitats and are frequently pressed to the limits of their physiological endurance must expend a greater proportion of their energy coping with the environment, and in addition will not be able to compete effectively with similar animals physiologically better adapted to that habitat. The result is a decrease in the energy available for reproduction. Animals which are pressed beyond their physiological limits die, and of course lose all of their reproductive potential.

Natural selection, favoring traits increasing reproductive success, would be expected to exert pressures for both maximizing energy acquisition and minimizing mortality (the latter especially in limpets, where reproductive output increases markedly with increasing size and age). The result is a balance between selection for exploitation of new resources by range extension, and selection for decreased risk of mortality by avoidance of marginally suitable range. Where the range overlaps with those of competing organisms, there is little energy to be gained by range expansion; the competing organisms presumably have a physiological advantage in that portion of the habitat. Under these conditions the balance of selection would be expected to favor behavioral adaptations resulting in occupation of physiologically more suitable portions of the range. However, where the habitat at and beyond the range limit of the species contains a resource unexploited by competing organisms, and opportunistic utilization of this resource increases reproductive success sufficiently to offset the risk of death, the balance of selection pressure will be shifted, from favoring stress avoidance to favoring range expansion.

This leads to the hypothesis that most species should be expected to be limited behaviorally, or by competition, to a range narrower than that in which their physiological tolerances would permit survival. Only those species whose range borders on an exploited resource might be expected to have extended their ranges to the limits of their physiological tolerances, and to be prevented from expanding them further by catastrophic mortality caused by physical factors. This hypothesis is supported by the data for *Acmaea*. The Zone II limpets *Acmaea pelta* and *A. t. scutum* are flanked above and below by other herbivorous gastropods, and do not appear to be limited by physical factors. On the other hand, the Zone I limpets *A. digitalis* and *A. persona* are flanked above in seep areas by a thick algal carpet, and appear to be exploiting this resource to the limit of their hypo-osmotic tolerances. In the remainder of the intertidal, *A. digitalis* and *A. scabra* are flanked above by a visible algal film and virtually no competitors; here they are limited by physical environmental factors causing desiccation.

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SUMMARY

1. This study tests the hypothesis that physical factors limit the ranges of five species of limpets (*Acmaea*) inhabiting the splash zone (Zone I) and upper mid-tidal (Zone II) of the Central California rocky shore. Two criteria are considered necessary to establish the hypothesis. First, interspecific differences in adaptation to physical factors must permit exploitation of different portions of the intertidal gradient. Secondly, physical conditions, by occasionally causing mortality, must set the limits of ranges at different levels depending upon the interspecific differences in physiological tolerances.

2. Dilution by winter rainwater runoff probably presents no osmotic threat to *Acmaea*. No pronounced differences are evident in either resistance to or tolerance of dilution. All five species tolerate immersion in fresh water for periods in excess of the maximum exposure expected in nature. Under exceptional circumstances some Zone I limpets may experience entrapment and lethal dilution in fresh water seeps.

3. Interspecific differences in tolerance to high temperatures are clearly correlated with solar heating occurring in the species' natural microhabitats. Thermal tolerances show no pronounced seasonal acclimation or intraspecific variation in limpets from different zones.

4. During three years, maximum field temperatures never exceeded the thermal tolerance of any of the limpets. Furthermore, all of the species tested, with the exception of *A. scabra*, seem to avoid maximally exposed microhabitats. It is concluded that high temperatures do not of themselves limit distributions of these limpets, but contribute to desiccation.

5. The Zone I limpets show higher desiccation tolerances and will tolerate drying conditions much longer than Zone II limpets. The limpets have no mechanism for avoiding the osmotic effects of desiccation; as expected, the Zone I limpets also have higher tolerances to hyperosmotic solutions and will tolerate elevated concentrations longer than will Zone II limpets.

6. Tolerances of desiccation and of hyperosmotic solutions in all 5 species are extremely high, ranging from about 70% to about 82% total water lost, and from 400% to 600% sea water.

7. Mortality during desiccation can be attributed entirely to the concentration of internal fluids resulting from evaporative water loss. Contrary to earlier reports, there are no increased water reserves in Zone I limpets. Thus high desiccation tolerances depend on adaptation to high electrolyte concentrations at the cellular level.

8. Desiccation rates of Zone I limpets are as low as $\frac{1}{10}$ those of Zone II limpets. The reduction is due almost entirely to a mucous diffusion barrier produced between the shell margin of Zone I species and the substratum. Non-"homing" *A. digitalis*, by virtue of the mucus sheet, have the same desiccation rate as "homing" *A. scabra*, suggesting that the principal advantage of homing may not be desiccation resistance alone.

9. Environmental conditions in Zone I exceed tolerances of the Zone II limpet species, and therefore potentially limit their ranges. However, behavioral adaptations apparently prevent upward migration and occupation of potentially lethal microhabitats. Hence, although differences in adaptation to drying conditions exist, the second criterion is not satisfied, and desiccation is not considered limiting for Zone II *Acmaea*.

10. The greater adaptation of Zone I limpets to drying conditions permits their exploitation of the high intertidal. Furthermore, desiccation does kill *A. digitalis* and *A. scabra* high in Zone I, especially small individuals, during periods of unusually warm weather or reduced splash. Thus, desiccation mortality does limit upper ends of intertidal ranges and apparently influences age structure in populations of these species.

11. The hypothesis is advanced that in general, range limits of animal popula-

tions should not be expected to be limited by physical factors, but by behavior, competition, or some other biotic factor. Only where the range borders on an unexploited resource would selection pressure favor range expansion to the limits of physiological tolerances. The data presented for *Acmaea* support this hypothesis: the Zone II species, with ranges overlapping those of the Zone I limpets above, are limited by behavior; the Zone I species, bordered above by a visible algal film, are limited by desiccation.

LITERATURE CITED

Abe, N., 1931. Ecological observations on *Acmaea dorsuosa* Gould. *Sci. Rep., Tohoku Imp. Univ. (Sendai)*, **6**: 403–427.

Abe, N., 1940. The homing, spawning, and other habits of a limpet, *Siphonaria japonica* Donovan. *Sci. Rep., Tohoku Imp. Univ. (Sendai)*, **15**: 59.

Allanson, B. R., 1958. Some aspects of the molluscan genus *Siphonaria* in South Africa. *Port. Acta Biol.*, (B)**6**: 179–212.

Arnold, D. C., 1957. The response of the limpet *Patella vulgata* L. to water of different salinities. *J. Mar. Biol. Ass. U. K.*, **36**: 121–128.

Barnes, H., and M. Barnes, 1964. Some relations between the habitat, behavior, and exposure to air of the high level intertidal cirripede *Chthamalus depressus* (Poli). *Helgolaender Wiss. Meeresunters.*, **10**: 19–28.

Baldwin, S., 1968. Manometric measurements of respiratory activity in *Acmaea digitalis* and *Acmaea scabra*. *Veliger*, **11** (Suppl.): 79–82.

Bock, C. E., and R. E. Johnson, 1967. The role of behavior in determining the intertidal zonation of *Littorina planaxis* Philippi, 1847, and *Littorina scutulata* Gould, 1849. *Veliger*, **10**: 42–54.

Boyle, P. R., 1969. The survival of osmotic stress by *Sypharochiton pelliserpentis*. *Biol. Bull.*, **136**: 154–165.

Boyle, P. R., 1970. Aspects of the ecology of a littoral chiton, *Sypharochiton pelliserpentis*. *N. Z. J. Mar. Freshwater Res.*, **4**: 364–384.

Broekhuysen, G. J., 1940. A preliminary investigation on the importance of desiccation, temperature, and salinity as factors controlling the vertical zonation of certain intertidal marine gastropods in False Bay, South Africa. *Trans. Roy. Soc. S. Afr.*, **128**: 255–291.

Brown, A. C., 1960. Desiccation as a factor influencing the distribution of some South African Gastropoda from intertidal rocky shores. *Port. Acta Biol.*, (B)**8**: 11–23.

Bursell, E., 1959. The water-balance of Tsetse flies. *Trans. Ent. Soc. London*, **111**: 205–235.

Colgan, N., 1910. Notes on the adaptability of certain littoral Mollusca. *Irish Natur.*, **19**: 127–133.

Cook, A., O. S. Bamford, J. D. B. Freeman and D. J. Teideman, 1969. A study on the homing habit of the limpet. *Anim. Behav.*, **17**: 330–339.

Das, S. M., and G. Seshappa, 1948. A contribution to the biology of *Patella*: On population distribution and sex proportions in *Patella vulgata* L. at Cullercoats, England. *Proc. Zool. Soc. London*, **117**: 653–662.

Davies, P. S., 1969. Physiological ecology of *Patella*. III. Desiccation effects. *J. Mar. Biol. Ass. U. K.*, **49**: 291–304.

Davies, P. S., 1970. Physiological ecology of *Patella*. IV. Environmental and limpet body temperatures. *J. Mar. Biol. Ass. U. K.*, **50**: 1069–1077.

Edelstam, C., and C. Palmer, 1950. Homing behavior in Gastropods. *Oikos*, **2**: 259–270.

Evans, R. G., 1948. The lethal temperatures of some common British littoral molluscs. *J. Anim. Ecol.*, **17**: 165–173.

Foster, B. A., 1971. Desiccation as a factor in the intertidal zonation of barnacles. *Mar. Biol.*, **8**: 12–29.

Fraenkel, G., 1968. The heat resistance of intertidal snails at Bimini, Bahamas; Ocean Springs, Miss.; and Woods Hole, Mass. *Physiol. Zool.*, **41**: 1–13.

Frank, P. W., 1964. On home range of limpets. *Amer. Natur.*, **98**: 99–104.

FRANK, P. W., 1965a. The biodemography of an intertidal snail population. *Ecology,* **46**: 831–844.

FRANK, P. W., 1965b. Growth of three species of *Acmaea. Veliger,* **7**: 201–202.

FRITCHMAN, H. K., 1961. A study of the reproductive cycle in the California *Acmaeidae* (Gastropoda). Part IV. *Veliger,* **4**: 134–140.

FUNKE, W., 1964. Untersuchungen zum Heimfindeverhalten und Ortstreue von *Patella* L. (Gastropoda, Prosobranchia). *Zool. Anz.,* **28**(Suppl): 411–418.

GALBRAITH, R. T., 1965. Homing behavior in the limpets *Acmaea digitalis* and *Lottia gigantea. Amer. Midland Natur.,* **74**: 245–246.

GOWANLOCH, J. N., AND F. R. HAYES, 1926. Contributions to the study of marine gastropods. I. The physical factors, behavior, and intertidal life of *Littorina. Contrib. Can. Biol. Fish.,* **3**: 133–166.

GUNTER, G., 1957. Temperature. *Geol. Soc. Amer., Mem.,* **67**(1): 159–184.

HANDBOOK OF CHEMISTRY AND PHYSICS, 1958. (40th edition) Chemical Rubber Publishing Company, Cleveland, Ohio.

HARDIN, D. D., 1968. A comparative study of lethal temperatures in the limpets *Acmaea scabra* and *Acmaea digitalis. Veliger,* **11**(Suppl.): 83–87.

HAVEN, S. B., 1970. Niche differences in the intertidal limpets *Acmaea scabra* and *A. digitalis* (Gastropoda) in Central California. *Veliger,* **13**: 231–248.

HAVEN, S. B., 1973. Competition for food between the intertidal gastropods *Acmaea scabra* and *Acmaea digitalis. Ecology* **54**: 143–151.

HELM, M. M., AND E. R. TRUEMAN, 1967. The effect of exposure on the heart rate of the mussel, *Mytilus edulis. Comp. Biochem. Physiol.,* **21**: 171–178.

HEWATT, W. G., 1940. Observations on the homing limpet, *Acmaea scabra* Gould. *Amer. Midland Natur.,* **24**: 205–208.

HINTON, H. E., 1951. A new chironomid from Africa, the larvae of which can be dehydrated without injury. *Proc. Zool. Soc. London,* **121**: 371–380.

HODGKIN, E. P., 1959. Catastrophic destruction of the littoral fauna and flora near Fremantle, January, 1959. *West Australian Naturalist,* **7**: 6–11.

HUNTSMAN, A. G., AND M. I. SPARKS, 1924. Limiting factors for marine animals. III. Relative resistance to high temperatures. *Contr. Can. Biol.,* **2**: 97–114.

KENSLER, C. B., 1967. Desiccation resistance of intertidal crevice species as a factor in their zonation. *J. Anim. Ecol.,* **36**: 391–406.

KESSEL, M. M., 1964. Reproduction and larval development of the prosobranch mollusc, *Acmaea testudinalis* (Müller). *Biol. Bull.,* **127**: 294–303.

KINGSTON, R. S., 1968. Anatomical and oxygen electrode studies of respiratory surfaces and respiration in *Acmaea. Veliger,* **11**(Suppl.): 73–78.

LEIGHLY, J., 1937. A note on evaporation. *Ecology,* **18**: 180–198.

LENT, C. M., 1968. Air-gaping by the ribbed mussel, *Modiolus demissus* (Dillwyn): effects and adaptive significance. *Biol. Bull.,* **134**: 60–73.

MCALISTER, R. O., AND F. M. FISHER, 1968. Responses of the false limpet, *Siphonaria pectinata* Linnaeus (Gastropoda, Pulmonata) to osmotic stress. *Biol. Bull.,* **134**: 96–117.

MACHIN, J., 1965. Cutaneous regulation of evaporative water loss in the common garden snail *Helix aspersa. Naturwissenschaften,* **52**: 18.

MARKEL, R. P., 1971. Temperature relations in two species of tropical west American littorines. *Ecology,* **52**: 1126–1130.

MICALLEF, H., AND W. H. BANNISTER, 1967. Aerial and aquatic oxygen consumption of *Monodonta turbinata* (Mollusca, Gastropoda). *J. Zool.,* **151**: 479–482.

MILLER, A. C., 1968. Orientation and movement of the limpet *Acmaea digitalis* on vertical rock surfaces. *Veliger,* **11**(Suppl.): 30–44.

NEWELL, R. C., V. I. PYE AND M. AHSANULLAH, 1971. The effect of thermal acclimation on the heat tolerance of the intertidal prosobranchs *Littorina littorea* and *Monodonta lineata. J. Exp. Biol.,* **54**: 525–533.

NORTH, W. J., 1954. Size distribution, erosive activities, and gross metabolic efficiency of the marine intertidal snails, *Littorina planaxis* and *L. scutulata. Biol. Bull.,* **106**: 185–197.

ORTON, J. H., 1929. Observations on *Patella vulgata.* Part III. Habitat and habits. *J. Mar. Biol. Ass. U. K.,* **16**: 277–288.

Orton, J. H., 1933. Some limiting factors in the environment of the common limpet, *Patella vulgata. Nature,* **131**: 693–694.

Paine, R. T., 1971. Energy flow in a natural population of the herbivorous gastropod *Tegula funebralis. Limnol. Oceanogr.,* **16**: 86–98.

Peterson, M., and F. C. Duerr, 1969. Studies on osmotic adjustment in *Tegula funebralis* (Adams 1854). *Comp. Biochem. Physiol.,* **28**: 633–644.

Pierce, S. K., 1970. The water balance of *Modiolus* (Mollusca: Bivalvia: Mytilidae): osmotic concentrations in changing salinities. *Comp. Biochem. Physiol.,* **36**: 521–533.

Pieron, H., 1909. Contribution a la biologie de la *Patella* et de la *Calyptrée:* le sens du retour et la mémoire topographique. *Arch. Gén. Zool. Exp. Paris,* **41**: 18.

Pilgrim, R. L. C., 1953. Osmotic relations in molluscan contractile tissues. I. Isolated ventricle-strip preparations from lamellibranchs. *J. Exp. Biol.,* **30**: 297–317.

Read, K. R. H., and K. B. Cumming, 1967. Thermal tolerance of the bivalve molluscs *Modiolus modiolus* L., *Mytilus edulis* L., and *Brachidontes demissus* Dillwyn. *Comp. Biochem. Physiol.,* **22**: 149–156.

Richardson, F., 1934. Diurnal movements of the limpets *Lottia gigantea* and *Acmaea persona. J. Entomol. Zool.,* **26**(4): 53–55.

Ricketts, E. F., and J. Calvin, 1962. *Between Pacific Tides* (4the ed., revised by Joel W. Hedgpeth) Stanford University Press, Stanford, California, 516 pp.

Russell, E. S., 1907. Environmental studies on the limpet. *Proc. Zool. Soc. London,* **1907**(2): 856–870.

Sandison, E. E., 1966. The oxygen consumption of some intertidal gastropods in relation to zonation. *J. Zool.,* **149**: 163–173.

Sandison, E. E., 1968. Respiratory response to temperature and temperature tolerance of some intertidal gastropods. *J. Exp. Mar. Biol. Ecol.* **1**: 271–281.

Segal, E., 1956a. Adaptive differences in water-holding capacity in an intertidal gastropod. *Ecology,* **37**: 174–178.

Segal, E., 1956b. Microgeographic variation as thermal acclimation in an intertidal mollusc. *Biol. Bull.,* **111**: 129–152.

Segal, E., and P. A. Dehnel, 1962. Osmotic behavior in an intertidal limpet, *Acmaea limatula. Biol. Bull.,* **122**: 417–430.

Shotwell, J. A., 1950a. Distribution of volume and relative linear measurement changes in *Acmaea,* the limpet. *Ecology,* **31**: 51–61.

Shotwell, J. A., 1950b. The vertical zonation of *Acmaea,* the limpet. *Ecology,* **31**: 647–649.

Southward, A. J., 1958. Note on the temperature tolerances of some intertidal animals in relation to environmental temperatures and geographical distribution. *J. Mar. Biol. Ass. U. K.,* **37**: 49–66.

Sutherland, J. P., 1970. Dynamics of high and low populations of the limpet, *Acmaea scabra. Ecol. Monogr.,* **40**: 169–188.

Test, A. R., 1945. Ecology of California *Acmaea. Ecology,* **26**: 395–405.

Thorne, M. J., 1969. Studies on homing in the chiton *Acanthozostera gemmata. Aust. J. Mar. Freshwater Res.,* **19**: 151–160.

Thorpe, W. H., 1963. *Learning and Instinct in Animals.* [2nd ed.] Methuen, London, 558 pp.

Thorson, T. B., and A. Svihla, 1943. Correlation of the habitats of amphibians with their ability to survive the loss of body water. *Ecology,* **24**: 374–381.

Villee, C. A., and T. C. Groody, 1940. The behavior of limpets with reference to their homing instinct. *Amer. Midland Natur.,* **24**: 190–204.

Webber, H. H., and P. A. Dehnel, 1968. Ion balance in the prosobranch gastropod, *Acmaea scutum. Comp. Biochem. Physiol.,* **25**: 49–64.

Wells, M. M., 1917. Behavior of limpets with particular reference to the homing instinct. *J. Anim. Behavior,* **7**: 387–395.

ABSTRACTS OF PAPERS PRESENTED AT THE MARINE BIOLOGICAL LABORATORY

Abstracts are arranged alphabetically by first author. Author and subject references will also be found in the regular volume index, appearing in the December issue.

GENERAL SCIENTIFIC MEETINGS

AUGUST 22–25, 1973

Properties of the gating currents of sodium channels. CLAY M. ARMSTRONG AND FRANCISCO BEZANILLA.

Charged gating structures of the sodium channels move after changes of the membrane potential (V_m), opening the sodium channels and in the process causing measurable "gating currents." We have studied these currents (after eliminating ionic current) and we describe here some of their properties and evidence linking them to the sodium channels. Preceding and overlapping the turn-on of g_{Na} there is an outward gating current that decays approximately exponentially. The rate constant is about 3 $msec^{-1}$ at $V_m = 0$ mV, and 6 $msec^{-1}$ at 60 mV (2° C); *i.e.*, kinetics are faster at more positive V_m. At the end of the pulse there is an inward current as the gating structures close. Gating current and sodium current have the same rate constant at pulse-end. This forces us to conclude that g_{Na} is not governed by several independent gating particles as it is in the m^3 formulation of Hodgkin and Huxley. (If gating currents were due to the movement of charged *m* particles, g_{Na} would decay three times faster than the gating current.) Inactivation of g_{Na} does not affect the gating current at pulse-end: amplitude is almost the same after a 1 msec or a 3 msec pulse. Tetrodotoxin does not alter the gating currents, but they are reversibly inhibited by two procedures that block g_{Na}. These are steady depolarization for a few minutes; and the addition of 10 mM $ZnCl_2$ to the internal perfusate (see the abstract by Begenisich and Lynch). For large depolarizations there is a small and slow component of gating current that we tentatively identify with gating of the K channels.

Dark adaptation of lobster. STEPHEN N. BARNES AND TIMOTHY H. GOLDSMITH.

Dark adaptation of the lobster *(Homarus)* was measured, using the electroretinogram (ERG), which in decapod crustacea is dominated by receptor potentials. Animals were immobilized in an aerated sea water bath which could be maintained at any desired temperature between 1° C and ambient. Temperature of the eye followed the bath to within 1–2° C and could be regulated to within a fraction of a degree. The animal was kept at least 2 hours in the dark at the experimental temperature before measurements began. Light adaptation was effected with a single intense white strobe flash, which converts as much as 78% of the rhodopsin to metarhodopsin. Migration of screening pigment could not be detected at 4° C nor during the first 10 minutes after a flash at room temperature. Changes in threshold were calculated from responses to test flashes during recovery, by referring to a response-energy function measured in the dark-adapted state.

To test whether photons absorbed by metarhodopsin have a physiological effect, test flashes were alternately blue (473 nm) and green (540 nm), wavelengths that are absorbed to different extents by metarhodopsin but about equally by rhodopsin. If, for example, rhodopsin and metarhodopsin were equally effective in excitation, displacement of sensitivity by the adapting flash should be 0.3 log units greater for the green test light than for the blue. The dark adaptation curves generated by these test flashes were identical, however, indicating that the spectral sensitivity of the eye was not altered by the adapting flash and that light absorbed by metarhodopsin does not excite or inhibit the receptor.

The course of dark adaptation was the same at 10°, 15°, and 20° C. Threshold was raised more than 1.3 log units by the adapting flash; it returned to within 0.5 log units in 2 minutes. At 5° C, on the other hand, dark adaption was much slower, returning to 0.5 log units in 12 minutes.

Supported by USPHS grant EY-00222 and a postdoctoral fellowship to S. N. B.

Reversible blockage of ionic currents by internal Zn. Ted Begenisich and Carl Lynch.

Squid giant axons were voltage-clamped and internally perfused with solutions containing the divalent cation Zn. The peak early current (I_p) was reversibly reduced by 10 mM Zn to 10% of its original value with no increase in leakage current. The steady-state current (I_{ss}) at first appeared to be even further depressed but upon subsequent examination using 1 mM Zn, it was found that I_{ss} was much slower. That is, the half-time of rise of I_{ss} ($t_{\frac{1}{2}}$) was increased by about a factor of five in 1 mM Zn. This effect was reversible.

Observations on current-clamped axons showed that internal Zn causes a reversible depolarization of the membrane potential. This may be due to an increase in the ratio of resting sodium and potassium permeabilities, as indicated by changing the external sodium concentration and by application of TTX.

The slowing effect on I_{ss} seen in voltage-clamped axons could be observed with Zn concentrations as low as 50 µM, and with either fluoride or glutamate as the internal anion. Hyperpolarizing prepulses caused a decrease in $t_{\frac{1}{2}}$ in Zn treated axons in contrast to the usual increase in $t_{\frac{1}{2}}$ in the absence of Zn.

Experiments by Francisco Bezanilla and Clay Armstrong have shown that Zn acts by reversibly interfering with the gating currents of excitable channels.

Effects of algal extracellular products on marine bacteria. Wayne Bell and Ralph Mitchell.

During log phase growth in the presence of ^{14}C-bicarbonate, the diatom *Skeletonema costatum* (SKEL) released 7–8% of its total photosynthate as extracellular C. When inoculated into a radioactive filtrate from a SKEL culture containing 620 µgC/l, two marine bacterial isolates showed a 40-fold difference in ability to take up labelled extracellular products: 0.4 µgC/l/hr for isolate 7697 (a spirillum) and 16 µgC/l/hr for isolate HNY (a pseudomonad) for populations of 10^5 cells/ml. These divergent uptake rates were then used in examining the behavior of the bacteria in SKEL cultures. In algal batch culture, HNY grew throughout the SKEL growth curve, while isolate 7697 showed a drop in population during algal log phase, recovering only late in stationary phase where algal cell lysis was obvious. In algal continuous culture, HNY rapidly increased to attain a steady state of 4000×10^4 cells/ml, but washed out to a steady state of 50×10^4 cells/ml in the absence of SKEL. Under the same conditions, viable cells of 7697 decreased from an inoculum of over 10^7 cells/ml at a rate much greater than the dilution rate, finally recovering to a steady state of only 20×10^4 cells/ml, the same as attained by controls cultured in the absence of SKEL. Since the data suggest marine bacteria differ in their ability to respond to the extracellular products, developing algal blooms may be accompanied by a physiologically specific "phycosphere" bacteria flora, while broad-spectrum stimulation of microbial activity should be restricted to periods of bloom decomposition when organics are released by lysing algal cells.

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Directionality of assembly of chick brain tubulin onto sea urchin flagella microtubules. Lester I. Binder and Joel L. Rosenbaum.

Earlier studies in our laboratory on flagellar regeneration in *Chlamydomonas* showed that the flagella elongated by the addition of material to their distal (tip) ends. Here, studies are reported on the directionality of microtubule assembly *in vitro* when pieces of sea urchin sperm axonemes were used as "seeds" for the assembly of chick brain tubulin. Chick brain tubulin subunits were purified by assembly at 37° C and dis-assembly at 4° C using the methods described by Weisenberg (1972, *Science,* **177**: 1104) for the assembly of rat brain microtubules *in vitro.* Sea urchin axonemes were isolated by modifications of methods published by Gibbons (1972, *J. Cell Biol.,* **54**: 75). When the tubulin subunits were incubated with pieces of axonemes in the presence of EGTA, GTP and Mg, negative staining and electron microscopy showed that neurotubules had assembled onto the A and central tubules and to a limited extent onto the B tubules of the axonemes. Neurotubules assembled onto both ends of the axoneme, although assembly onto the distal end was much faster than that onto the proximal end at any given subunit concentration. Furthermore, we were able to show that the rate and amount of neurotubule assembly onto each end were dependent on subunit concentration. Thus, the brain tubulin concentration could be lowered to the point where assembly occurred only onto the distal end of the axoneme; at higher subunit concentrations, assembly occurred onto both ends. The neurotubules were differentiated from the flagellar tubules by their dissociability with cold or calcium treatments, their differential staining with uranyl acetate, and because the neurotubules became several times longer than the pieces of axonemes. The results show that the tubulin assembly sites are conserved between organisms (sea urchin, chicken). Also, the uni-directionality of assembly seen during flagellar regeneration *in vivo* (distal growth) is not adhered to during assembly of this heterologous system *in vitro* where, at high subunit concentrations, assembly was bi-directional.

This study was performed in the Fertilization and Gamete Physiology Training Program at the Marine Biological Laboratory (NIH grant 5-T01-HD-00026-12).

Conditions for attachment of single cells released from mechanically-disrupted thalli of Prasiola stipitata *Suhr.* SCOTT E. BINGHAM AND JEROME A. SCHIFF.

Single cells released from thalli of *Prasiola stipitata* Suhr by hand homogenization attach to substrates by secreting a mucilagenous material and, through successive divisions, eventually develop into new plants. The proportion of cells which finally attach approaches 100% when cell concentrations are below 6.0×10^5 cells/ml; above this concentration the rate decreases rapidly with increasing cell number due, at least in part, to cell clumping. At optimal concentrations in the dark, attachment is dependent on temperature; at 4° C the attachment rate is virtually zero while at 23° the number attached approaches 100% within 24 hours. In the light attachment is much less temperature dependent, good attachment being achieved at 4°. Treatment with 10^{-5} M DCMU, an inhibitor of photosynthesis, reduces the attachment rate at 4° in the light to that found in the dark at the same temperature. Thus energy from either photosynthesis or respiration can be utilized for attachment. Treatment with cycloheximide or chloramphenicol (0.5 g/l) added at the beginning of the attachment assay allows only 40–50% attachment. Preincubation with cycloheximide for 12 hours at 4° in darkness inhibits attachment nearly completely when such cells with antibiotic are placed in the light. The same experiment with chloramphenicol results in a 40–75% inhibition of attachment. Thus protein synthesis seems to be required for attachment to proceed and preincubation with cycloheximide is necessary to avoid an escape from the antibiotic action during initial hours of attachment. Cells obtained from plants collected from Manomet in June and Nobska and Penikese in late July completed attachment within 15 hours while mid-July Manomet material began attachment only after 20 hours and took 28–30 hours to complete it.

This work was supported by grants GB-37149X and GB-25920 from NSF and GM-14595 from NIH.

Some chemical and physical properties of Fundulus *hemoglobins.* THOMAS A. BORGESE, JOSEPH M. DUVA AND DENNIS POWERS.

Preparative column and analytical disc-gel electrophoresis were used to investigate some of the properties of hemoglobin from the killifish, *Fundulus heteroclitus.* The purity of column separated hemoglobins, (I to IV, in order of increasing anodal mobility), concentrated by passage through Sephadex G-25, was established by re-electrophoresis on acrylamide disc-gels. Sedimentation velocity studies on fresh, unfractionated hemoglobin and hemoglobins II and III show a single peak with a sedimentation coefficient of 4.23 corresponding to a hemoglobin tetramer with a molecular weight of approximately 65,000. Storage of hemoglobin III at 4° C results in the appearance of at least two peaks with sedimentation coefficients of 11.89 and 4.34 for the heavier and lighter components, respectively, and suggests varying degrees of aggregation of the hemoglobin tetramer. Electrophoresis of stored, isolated hemoglobins shows that a mixture of hemoglobins II and III can give rise to all four hemoglobins as judged from their electrophoretic mobilities and provides evidence for dissociation and recombination of the hemoglobin chains. Globin chain analysis of unfractionated hemoglobin indicates the presence of three chains, *A, B* and *C*. Chain *A* is common to all hemoglobins, is the fastest moving cathodal component at pH 2.5 and is usually present in the highest proportion. The disproportionate amount of chain *A* suggests a remote possibility that it may consist of two or more similar chains unresolved by the present technique of gel electrophoresis. Hemoglobins I and IV consist of chains *AB* and *AC*, respectively, while hemoglobins II and III contain chains *A, B* and *C* with the proportion of *B* to *C* reversed in these two hemoglobins. The *in vivo* administration of iron-59 to single killifish shows a differential rate of incorporation into hemoglobin. The highest specific activity is associated with hemoglobin I after 22 hours or 8 days at 22° C. This labelling pattern is not changed after 24 hours at 5° C for cold-adapted fish.

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Bioluminescent characteristics of the ophiuroid, Ophiopsila californica. PAUL H BREHM, JAMES G. MORIN AND GEORGE T. REYNOLDS.

The spatial distribution of luminescent effectors was determined in *Ophiopsila californica*, a southern California ophiuroid, using image intensification techniques. The disc lacks luminescent capability except for (1) the oral spines present in the ambulacra and (2) the radial shields which are luminescent in some individuals. All arm segments show identical distribution of luminescence. Specific arm parts were surgically isolated from the arm and then tested for luminescence under image intensification by (1) electrical stimulation or (2) adding isotonic potassium chloride. These techniques revealed that all spines are luminescent with the ventral-most pair producing the most light. In all cases the entire spine is capable of light emission but the base is considerably brighter than the tip. Both the large and small tentacular scales luminesce. The smaller tentacular scale is almost entirely luminescent, while the larger scale shows uniform luminescence only at the basal third with fine channels showing propagated luminescence to about midway up the scale. The ventral and lateral plates are capable of uniform luminescence over their entire surface. The dorsal plates are not luminescent. The net result of this distribution of luminescence is a greater intensity of luminescence from the oral side of the arm than the aboral side. The tube feet, arm musculature and vertebral ossicles are not luminescent.

The luminescence is complexly controlled by neural propagation along the arms. Variable conduction velocities, backing and filling of luminescent sites along the arm, and the distinct capacity for sites within and between segments to fire independently makes physiological analysis difficult. Alternating pigmented bands along the arms are associated with different thresholds of luminescence; tan bands having a lower threshold than brown bands. Luminescence frequently occurs from the spines and plates along one side of the arm while the other side remains dark. This observation suggests that distinct tracts innervate contralateral sides of the arm separately from one another.

By spectroscopic techniques coupled to image intensification the luminescent emission spectrum was determined to be broad with (1) a range from about 460 nm to about 600 nm, (2) a peak emission between 525 and 530 nm, and (3) a shoulder at about 485 nm.

Ecological aspects of photoheterotrophic and heterotrophic growth on amino acids. LINDA BREWER AND JANE GIBSON.

The algal mat at Sippewisset Marsh near Woods Hole, composed of intensely colored layers of photosynthetic bacteria underlying a dense mat of blue-greens, provides an ideal habitat for the investigation of possible nutritional relationships between marine strains of these two types of procaryote. Since blue-greens are known to release both amino acids and small peptides into the medium, utilization of amino acids as a sole source of carbon for anaerobic growth of bacteria was investigated by liquid enrichment culture and direct plating of dilutions on solid media.

Liquid enrichments gave rise to a succession of bacterial types, in which small motile bacteria preceded the development of pigmented photosynthetic organisms. In direct plating, no pigmented colonies developed, but heterotrophic organisms were found in greater numbers near the algal mat than in the lower layers. In a total of 7 isolates of photosynthetic bacteria only one grew in amino acid medium. These results suggested that the action of heterotrophic bacteria was necessary in order to make the amino acids medium suitable for growth of photosynthetic types. This possibility was tested by inoculating these 7 photosynthetic isolates into tubes of medium which had first been used for growth of the heterotrophic organisms and then passed through a membrane filter. All photosynthetic organisms grew in this medium. In addition, a mineral salts medium which did not permit growth of bacterial isolates when freshly prepared, did support the development of the heterotrophic, but not the photosynthetic, organisms after *Oscillatoria* had been growing in the medium and removed by filtration. These results indicate that materials leaked during the growth of the blue-green algae can support the growth of heterotrophic, but not photosynthetic, isolates from the marsh, and that the activity of heterotrophic anaerobes can make an amino acid medium, in itself unsuitable for photosynthetic isolates, acceptable for their growth. Photosynthetic bacteria in the mat may therefore be indirectly stimulated by leakage products from the algae, but more complex growth experiments are necessary to test this hypothesis.

Isolation of a dynein-like protein associated with brain microtubules. Roy G. Burns and Thomas D. Pollard.

Dogfish brain contains a high molecular weight polypeptide which comigrates on SDS-polyacrylamide gels with the larger of the two dyneins, the microtubule-associated ATPase of sperm tail flagella. Extrapolation of plots of 8 reduced and alkylated protein standards (20,000–200,000 dalton range) or plots of unreduced polymers of myosin, gamma globulin and BSA indicate that the subunit molecular weight of the brain protein and the larger flagellar dynein is 380,000, while that of the smaller flagellar dynein is 360,000. The brain component is $<1\%$ of the total protein. On fractionation of a 100,000 *g* supernatant on a 4% agarose column there are several peaks of Mg^{++}ATPase activity in the high molecular weight range. One peak ($K_d = 0.3$, Stokes radius about 12 nm, like a fraction of *Spisula* sperm dynein) is enriched with the 380,000 dalton protein (4%), but also contains 34% tubulin.

Intact microtubules isolated in hexylene glycol have a Mg^{++}ATPase activity of about 1 μmole $P_i \cdot min^{-1} \cdot mg^{-1}$. A similar activity (2 μmoles $P_i \cdot min^{-1} \cdot mg^{-1}$) is associated with microtubules reassembled *in vitro* following warming to 25° C and pelleting the reassembled microtubules. Less ATPase is pelleted if microtubule assembly is inhibited with calcium or colchicine. About 50% of the ATPase activity is lost on dissociation of the microtubules with 0.5 M KCl or cold treatment. This suggests that the microtubule-associated ATPase activity is partially dependent upon the integrity of the microtubules. Gel filtration (4% agarose, 4° C) of the dissociated microtubules separates the residual ATPase activity ($K_d = 0$) from tubulin. The ATPase fraction is enriched in polypeptides with molecular weights of 80,000, 140,000 and 170,000. The fraction which elutes at $K_d = 0.3$ has little or no ATPase activity, but is enriched in the 380,000 dalton protein (36%, representing a 100 fold purification), contaminated with an equal amount of tubulin and several other proteins.

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Behavioral analysis of visual communication in the firefly Photinus greeni. James Case and John Buck.

In courtship the male of *P. greeni* emits flashes in pairs about 1400 msec apart at 22°. After the second flash of each pair the female gives a single flash of about 900 msec latency. The critical element in this communication system is the timing of the flashes. We have taken advantage of this simple code to measure the visual sensitivity of the female as well as other aspects of her behavior that influence the efficiency of communication. The threshold of dark-adapted females to 250 msec flashes of light from a Sylvania glow modulator tube is 8×10^{-11} μW cm^{-2} for immobilized females forced to look directly at the signal *vs.* 8×10^{-10} for females free to assume random body orientations. Peak light emission by males was 9×10^{-3} μW cm^{-2} (560 nm) and by females was 4×10^{-4}. From these data and the dimensions of the female's eye we estimate the effective visual communication range to be of the order of 5 m, which is compatible with field observations. The female responds normally to a flash pair irrespective of whether the two flashes are delivered exclusively to the same eye or to both eyes serially. She senses the direction of the male's signal from the direction of the second flash of the pair and reacts by turning her light organ in that direction about 200 msec before flashing. She is, however, unable to discriminate directionally between signal pairs, as evidenced by the inhibitory effect of a single out-of-phase flash to one eye while the other receives a normal signal pair.

This firefly is thus a useful object for behavioral analysis of the insect visual system since it responds unequivocally to any properly timed signal pair, and at very low intensities.

J. F. C. was supported by an N.S.F. grant.

Electron microscopic study on membrane junctions of Arbacia punctulata *blastomeres.* DONALD C. CHANG AND BJORN A. AFZELIUS.

Membrane junctions in early embryos of American sea urchin, *Arbacia punctulata*, were studied by electron microscopy. The cells were found to be connected by septate junctions from the four-cell stage on. These junctions were located at the external end of the intercellular boundaries and thus close to the hyaline layer. Unlike those found in some adult epithelia, these septate junctions are narrow and composed of only two to five septa (60 Å thick and 200 Å wide). Earlier studies have revealed similar kinds of septate junctions in other species of sea urchins but only in later stages of development (late blastula or gastrula).

The physiological function of the septate junction can be one or more of the following: (1) Adhesion: the cells of the normal sea urchin embryo are held together by the fertilization membrane, the hyaline layer and possibly by cell junctions. As the blastomeres can be freed of fertilization membrane and hyaline layer and still retain their mutual connections, the septate junctions may function in cell adhesion. (2) Intercellular communication: it has been proposed that cells communicate through the septate junctions. No electrical coupling has, however, been found until at the 32-cell stage in the echinoderm embryo. The existence of septate junctions may thus not correlate with the onset of electrical communication. (3) Sealing: it can be inferred from the present data that septate junctions form a continuous band which covers the external openings of the intercellular space. This leads us to speculate that the intercellular fluid of the early embryo may be different from and sealed off from the external sea water. The sealing by the septate junctions then enables the embryo to establish and regulate its own environment and provides a pathway via which the cells can communicate by chemical means.

The most important function of the septate junction may be that of sealing.

This study was performed in the Fertilization and Gamete Physiology Research Training Program at the Marine Biological Laboratory (NIH 5-T01-HD00026-12).

Environmental adaptation of Fundulus heteroclitus *muscle and liver lactate dehydrogenase.* VERNON CLARK AND DENNIS A. POWERS.

Recent experiments have raised the possibility of comparing allotype kinetics as a function of environmental adaptation. However, one wonders how much molecular adaptation is the result of quantitative substrate or enzyme changes. We have adapted fish to 5°, 10°, 20° and 30° and shown that the quantities of lactate, glycogen and lactate dehydrogenase do not change with environmental temperature. However, pyruvate, glucose and ATP pools do change significantly. The kinetics of the enzymes suggest that molecular adaptation as well as allelic competition is a function of environmental temperature and influenced directly by the structural and functional properties of the enzymes.

Support was provided by: NSF grant #GB37548 and an Electric Power Research Institute grant #P407901 and NIH grant (GM00265-15) to the physiology course at MBL.

Evidence of 1-methyladenine synthesis by isolated starfish ovarian follicle cells in vitro. J. G. CLOUD AND A. W. SCHUETZ.

Previous studies have implicated ovarian follicular cells of the starfish as a source and site of synthesis of a meiosis inducing substance (1-methyladenine). The capacity of isolated follicle cells to synthesize 1-methyladenine *in vitro* was investigated with the use of radioactive precursor (^{3}H-methylmethionine). Detachment of follicle cells from oocytes was accomplished after mincing ovaries in calcium free sea water. Follicle cells were separated from oocytes and concentrated by differential centrifugation. Isolated follicle cells (0.5–1.0 g equivalents of ovarian tissue) were incubated in 2 ml of artificial sea water containing isotope. A rapid and concentration dependent uptake of radioactively labeled methionine by the follicle cells occurred *in vitro*. The amount of radioactivity retained by the follicle cells was less when calcium ions were present in the incubation mixture. Following incubation, follicle cells were removed and the supernatant was fractionated by means of gel filtration (Sephadex G-25–150). A peak of radioactivity, separate from methionine, was isolated from the supernatant which co-chromatographed with authentic radioactive 1-methyladenine and non-radioactive carrier 1-methyladenine as detected by bioassay. Increased amounts of the second component were observed when follicle cells were also exposed to starfish radial nerve factor. Radioactivity was also localized in the 1-methyladenine region when ^{3}H-adenine was incubated with follicle cells. The amount of radioactivity incorporated into the region of 1-methyladenine appeared to decrease during the course of the breeding season. The data suggest that ovarian follicle cells are viable and capable of synthesizing 1-methyladenine from methionine under *in vitro* conditions.

Research supported by a grant from the Population Council.

Calcium electrogenesis in skate electroreceptors. W. T. CLUSIN AND M. V. L. BENNETT.

Skate ampullary electroreceptors have two faces which are non-isopotential because they are part of a high resistance epithelium. The lumenal membrane produces action potentials which passively depolarize the basilar, secretory, membrane causing synaptic transmitter release. The secretory membrane is normally inexcitable. By electrically isolating the two surfaces of the ampulla, it is possible to synchronize the receptor action potentials so that true transmembrane potential changes can be recorded with extracellular electrodes. Using this technique we have obtained evidence that the lumenal membrane action potential is calcium-dependent. We have also demonstrated that the secretory membrane can be made to produce calcium action potentials when its delayed rectification is abolished by pharmacological antagonists. The lumenal membrane action potential has a duration of 100 msec and is associated with a conductance increase across the ampullary epithelium. It is insensitive to 10 μM TTX, but is abolished by Co^{++}. The secretory membrane produces action potentials only when it is exposed to high extracellular concentrations of Ca^{++}, Ba^{++}, or Sr^{++}. When Ca^{++} is used, TEA must be added, presumably to antagonize the potassium conductance. Like the physiological response, the secretory action potential is associated with a conductance increase across the epithelium. It may be evoked directly, by currents that depolarize the secretory membrane, or indirectly, by exciting the lumenal membrane. The presynaptic action potential is strictly analogous to calcium electrogenesis in squid giant synapse as described by Katz and Miledi. It is associated with a prolonged PSP in the nerve and is abolished by concentrations of Mn^{++}, Co^{++} or La^{+++} sufficient to block transmitter release. Applying these antagonists to the presynaptic membrane does not affect the Ca^{++} action potential in the lumenal face. This experiment supports the notion that only calcium channels near the synapse are involved in transmission.

W. T. C. is an NIH pre-doctoral trainee.

Changes in fluorescence of a squid giant axon during excitation, a demonstration. L. B. COHEN, B. M. SALZBERG AND H. V. DAVILA.

Changes in the fluorescence of a merocyanine dye bound to the axon were demonstrated. The fluorescence changes were potential dependent and provide a promising method for measuring membrane potential in the absence of electrodes.

Supported by grant number NS 08437 from the National Institute of Neurological Diseases and Stroke.

Isolation of Arbacia *sperm antigens important in fertilization.* CHRISTOPHER T. CORDLE AND CHARLES B. METZ.

Rabbit antisera inhibit the fertilizing capacity of *Arbacia* sperm. Such inhibition is not exclusively dependent upon the antisera's sperm agglutinating activity since papain digested non-aggluting antibody (Fab fragment) also inhibits the fertilizing capacity of the sperm. It evidently results from blocking one or more essential antigens.

Extracts of whole washed *Arbacia* sperm prepared by repeated freeze-thawing were examined for such antigens. The extracts were centrifuged at 80,000 $\times\ g$ for 40 min. The supernatant neutralized the fertilization inhibiting action of univalent antibody. This indicated one or more soluble "fertilization antigens" in the extract. Immunoelectrophoresis indicated that most of the antigenic material had a net negative charge.

The extract was fractionated using DEAE cellulose columns (DEAE in 0.01 M phosphate pH 7.5 with NaCl gradient elution). The elution profile (O.D. 280) showed seven major peaks. Seven pools were made from the column fractions, each pool was tested for neutralization of fertilization inhibiting action of univalent antibody. Only pools V and VI showed such activity.

SDS gel electrophoresis under reducing conditions showed that pools V and VI contain one prominent fast moving protein band not found in the other pools. This protein may be the antigen involved in the inhibition process. None of the pools had antifertilizin activity as judged by their inability to precipitate egg jelly and inability to neutralize the egg jelly precipitating action of antifertilizin prepared by heating sperm.

It is concluded that: (1) Freeze-thaw extracts of *Arbacia* sperm contain soluble fertilization antigen(s). (2) DEAE chromatography may be used to purify the fertilization antigen(s). (3) SDS gel electrophoresis and immunodiffusion experiments indicate that only a few antigens were involved. (4) The antigen(s) involved is not antifertilizin. (5) The results do not exclude the possibility of additional "insoluble" fertilization antigens.

This study was performed in the Fertilization and Gamete Physiology Research Training Program at the Marine Biological Laboratory (NIH grant 5-T01-HD00026–12).

A reduction in water permeability in response to a dilute medium in the stenohaline crab Libinia emarginata *(Brachyura, Majidae).* JOHN C. CORNELL.

An adaptive reduction in diffusive water permeability has been shown to occur in several osmoregulating crustaceans (Rudy, 1967, *Comp. Biochem. Physiol.*, **22**: 581; Smith, 1967, *Biol. Bull.*, **133**: 643). The site of the reduction in water permeability has been shown to be the gills (Capen, 1972, *J. Exp. Zool.*, **182**: 307). It was decided to examine water permeability in *Libinia emarginata* an osmoconforming crab.

Adult male animals between 150 and 250 g were obtained from the supply department at the Marine Biological Laboratory at Woods Hole. All of the experiments described below were carried out at 20–21° C. Large net movements of water were indicated when animals were transferred from 100 to 80% SW. Maximum weight gains (0.5%, $N = 20$) and increases in urine production rates (600%, $N = 2$) occurred one hour after transfer. The heart rate of 7 animals was observed to decrease from 55 to 35/min ($P < 0.05$) during this time. Since changes in circulation are known to effect diffusive water permeability, it was hypothesized that (1) *Libinia* would show a decrease in water permeability and (2) this decrease could be accounted for by the change in heart rate. The first part of this hypothesis was tested by measuring water permeability with D_2O. The rate constant K (hr^{-1}) for 16 animals in 100% SW+ 2.5% D_2O was 8.49. After a one hour exposure to 80% SW the same animals were placed in 80% SW + 2.5% D_2O and the value for K was found to be 5.96, indicating a 30% reduction ($P < 0.002$) in permeability. It was not possible to directly test the second part of the hypothesis. However, india ink was injected into the haemocoel and a rate of flow of 45 ml/hr was observed in the fourth gill of a 200 g animal. The fourth gills of 6 animals were isolated and perfused with a peristaltic pump which recirculated 2 ml of Ringer's through the gills. When the gills were placed in 100% SW + 2.5% D_2O and perfused at the rates of 25, 47 and 90 ml/hr, the Ringer's solution was found to be 6.3, 14.5 ($P < 0.001$) and 18.5% saturated with D_2O, respectively. This is believed to be the first report of a change in permeability in an osmoconforming animal and the first indication that changes in circulation may be responsible for at least part of the diffusive water permeability change in crustaceans.

I would like to acknowledge the Experimental Invertebrate Zoology Class for their support.

Glycosaminoglycans in lens capsules of dogfish. G. CREMER AND Z. DISCHE.

Mammalian lens capsules, typical basement membranes, contain non-fibrillary collagen, a polyhexuronide and two types of sialoglycoproteins. One solubilized with the collagen, the other, 10% of the total, is extracted only by β mercaptoethanol (ME). The topical distribution of this latter glycoprotein in the capsule suggests a correlation with the varying density of the collagen matrix and possibly elasticity of the capsule, a factor in visual accommodation in vertebrates except fishes. Here accommodation is obtained by changing the distance lens to retina. A different glycoprotein pattern might therefore prevail in fish capsules. This suggested to determine the presence and ratio in dogfish capsules of glycoprotein fractions corresponding to those in mammalian capsules. Capsules (0.6–1 g) stripped off from fresh dogfish lenses, washed with M/50 Tris buffer pH 7.4 were incubated twice at R.T. 16^h in a Tris buffer M/50 pH 7.4 containing 0.005 M $CaCl_2$, antibiotics and 400 units high purity collagenase (Worthington) per 10 ml. Each digest was centrifuged and the residues were washed. The second digestion residue was extracted 16^h at R.T. with 0.1 M ME pH 7.4 and the extract dialyzed. Hydroxyprolin, hexosamine and fucose were determined in the collagenase digests and the ME extract. The level of hydroxyprolin was much smaller in the second collagenase digest than in the first one. The solubilization of hexosamine paralleled that of hydroxyprolin. In the ME extract the amount of hexosamine was about $\frac{1}{3}$ of that extracted by collagenase. Only traces of hydroxyprolin were in this extract. The ratio fucose to hexosamine in the extract was 0.18. The situation is therefore similar to that in mammalian capsules.

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The use of enrichment techniques to study marine bacterial viruses. PAUL CURTIS AND KENNETH NEALSON.

Very little quantitative information is available concerning the distribution, numbers and ecological significance of marine bacterial viruses. We report here the use of quantitative enrichment techniques in the study of the occurrence and numbers of marine bacterial viruses in Eel Pond Harbor, Woods Hole.

Using standard enrichment culture techniques we first isolated two bacteriophage active against two different strains of marine luminous bacteria. Then, using these newly isolated phage, as well as a well defined phage (hv-1 and its marine host MAV) we demonstrated that a 16 tube dilution matrix valuable in determining, within a factor of 10, titers of phage ranging from 0.1 per ml to 5×10^3 per ml.

This matrix technique was then used to examine both Eel Pond water and sediment samples for the presence of phage active against twenty six different strains of luminous bacteria, both symbiotic and saprophytic (free living) from diverse origins. Six phage were isolated in the first attempt, all of them active against free living strains, and none against the symbionts. In all cases, the naturally existing titer of phage was approximately 10 per ml or less in the sea water, and 10-fold higher in the sediment samples.

Host range studies were performed on the six newly isolated phages, and indicated first, that at least five of the six are very likely unique phages, and second, that they have a wide variety of host ranges (from one to eight strains are sensitive), all confined to the free living strains in our collection.

Optical recording of neuronal impulses in the leech central nervous system. H. V. DAVILA, B. M. SALZBERG, AND L. B. COHEN.

Previous experiments showed that the fluorescence of a squid giant axon stained with a merocyanine dye increased by 0.1% during the action potential. Since the noise level was approximately 0.01%, action potentials in the giant axon could be monitored by measuring fluorescence intensity, in the absence of electrodes. A similar result has now been achieved in cell bodies of sensory neurons in segmental ganglia from the leech, *Hirudo medicinalis.*

The emitted light from a stained ganglion was collected by a 20× microscope objective. A pinhole in the objective image plane allowed the light from one cell to reach the photodetector, and blocked the light from the remainder of the ganglion. The selected cell was impaled with a potassium acetate filled microelectrode and was stimulated by passing current through the electrode. The fluorescence intensity changes that occurred during the action potential could be measured with a signal to noise ratio of about 5:1. When the pinhole was moved by one diameter so that the image of the stimulated cell did not fall on the photodetector, no signal could be measured.

We hope that it will be possible to expand the system to employ an array of detectors so that many neurons can be studied simultaneously. The ability to record electrical activity in a large number of cells at once would allow the construction of a detailed map of the functional connections within a ganglion, and, with additional experiments, should enhance our understanding of the neuronal basis of behavior and behavior modification.

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Aldehyde analogs and the mechanism of bacterial bioluminescence. ANATOL EBERHARD, DOTTI BENTLEY AND ROBERT SOLSKY.

Bacterial bioluminescence requires a long-chain aldehyde *in vitro.* The reaction was studied using analogs of aldehydes. In comparison to dodecanal, 1-deuterododecanal showed a kinetic isotope effect of 1.6 for the first order *in vitro* decay of luminescence after mixing reduced flavin mononucleotide, oxygen, luciferase and the aldehyde. As expected, however, the quantum yield for deuterododecanal was the same as that for dodecanal. Decyl nitrite was also found to yield light in the enzymic reaction, with a quantum yield of up to 30% of that for dodecanal. Bacterial mutants that do not emit light unless aldehyde is added to the medium emitted light *in vivo* when decyl nitrite was added, with a quantum yield greater than that for a similar quantity of aldehyde. These results support a mechanism for bacterial bioluminescence proposed earlier: reduced flavin and oxygen form a peroxy anion which attacks aldehyde. The resulting peroxy hemiacetal undergoes a reaction similar to the Baeyer-Villiger reaction, giving the corresponding carboxylic acid, hydroxide and excited oxidized flavin which subsequently emits a photon. Our results support this mechanism because an identical mechanism can be written with nitrite replacing aldehyde and because a deuterium isotope effect is expected if the Baeyer-Villiger step is rate determining.

Local adaptation in the ventral photoreceptor of Limulus. ALAN FEIN.

The lateral olfactory nerves containing the ventral photoreceptors were dissected free, desheathed, and placed in a small chamber containing sea water. The receptor potential was recorded intracellularly using a glass micropipette. An adapting spot of light (10 μm diameter) was positioned on one region (position A) of an individual photoreceptor. A second spot of the same size was positioned on the same photoreceptor either at the adapting spot (position A) or at a position (position B) 80 μm removed from the adapting spot (ventral photoreceptors typically have cross sections of 50 × 100 μm or larger).

Either flash illumination or continuous illumination with the adapting spot at position A raised the threshold (criterion response of receptor potential) at position A by approximately 1.4 log units. Whereas the threshold at position B was raised by less than 0.3 log units. Uniform illumination of the whole photoreceptor elevated the threshold at both A and B by approximately 1.4 log units.

These results indicate that light adaptation in this receptor is a local process which does not spread to unilluminated regions of the cell.

5-Hydroxytryptamine: an uptake mechanism in synaptosomes from the optic lobe of squid (Loligo pealei). JACK L. FELDMAN AND MICHAEL J. DOWDALL.

Uptake of (1–2-^{3}H)(N) 5-hydroxytryptamine (^{3}H-5-HT) in squid optic lobe synaptosomes, incubated in Tris buffered artificial sea water (pH = 7.4), was studied with millipore filtration. Uptake of 0.79 μM ^{3}H-5-HT was time and temperature dependent, with a maximum accumulation of radioactivity at about 30 minutes; 96% of the radioactivity at this time was present in an osmotically sensitive compartment. After 30 minute incubations, the minimum concentration ratios (inside:outside) were about 17:1, 150:1, and 340:1 at 0° C, 23° C and 32° C, respectively; this suggests the existence of an active uptake process working against a concentration gradient. Varying 5-HT concentrations (10^{-8} M–10^{-4} M) at 23° C showed the uptake system to be saturable with a K_m of 0.6–1.2 μM and V_{max} of 0.3 nmol/min/gram original tissue. These values are similar to those for the high affinity 5-HT uptake system in mammalian 5-HT rich striatum and hypothalamus. It is believed that this high affinity uptake is a property of serotoninergic nerve terminals; squid optic lobes are known to contain quantities of 5-HT comparable to the mammalian CNS tissue; therefore the present results further suggest that 5-HT is a neurotransmitter in the squid optic lobe.

Two structural analogues of 5-HT, d-lysergic acid diethylamide (LSD) and 2-brom-d-lysergic acid diethylamide (Br-LSD) had negligible effects on the uptake of 0.79 μM ^{3}H-5-HT in concentrations ranging from 10^{-7}-5×10^{-6} M, whereas 10^{-7} M chlorpromazine was seen to markedly inhibit uptake. Since LSD can effect serotoninergic transmission in molluscan heart at concentrations as low as 10^{-15} M, it seems unlikely that the nervous system dysfunction associated with LSD is mediated via presynaptic blockade of the high affinity 5-HT uptake system.

We thank Michael Greenberg for supplying 5-HT and analogues. M. J. D. thanks the Welcome Foundation for support. The Neurobiology course was supported in part by the Grass Foundation.

Preliminary studies of the skate's semicircular canal. ÅKE FLOCK AND MOÏSE H. GOLDSTEIN, JR.

Single unit recordings were obtained from nerve fibers innervating the crista ampularis of the skate *Raja erinacea* and *Raja ocellata*. A simple hydraulic system using a 1 μl syringe driven by hand or loudspeaker cone moved the cupula by volume displacement of the fluid in the semicircular canal. The skate was chosen for the size and accessibility of the inner ear and because the posterior ampulla can be dissected as an isolated preparation with long canal projections. A window discriminator allowed isolation of single unit activity. Some neural recordings were from the fibers innervating the horizontal canal crista in which case the inner ear remained in cartilage. It was possible to study either preparation for six hours or more. Response characteristics were obtained by plotting spikes during 10 sec volume displacements. The curves increased monotonically reaching a plateau for displacements of about 0.1 μl. Increases over spontaneous spike rate were easily apparent for displacements as small as 0.01 μl. Staining with Alcion blue made the cupula visible. Volume displacements of 0.1 μl yielded movements of about 75 μm for a midline midpoint on the cupula. The corresponding angular deflection of the cupula is about 6°. This movement is probably near maximum or surpasses the physiological range. A variety of patterns of spike discharges were observed. Some units showed tonic responses to excitatory displacements with elevated firing rates lasting several minutes and strong phasic components lasting less than 5 sec. Other units showed only phasic responses. Step displacements opposite the excitatory direction were always suppressive with tonic units taking up to ten minutes to regain their full spontaneous rate and sensitivity to small test excitations. The steady state firing pattern of some tonic units exhibited quite irregular intervals while others had more regular intervals.

We gratefully acknowledge support of the Grass Foundation and the Johns Hopkins University.

The occurrence and sulfation of kappa- and lambda-carrageenan in Chondrus crispus. F. FONG, J. DERR AND F. LOEWUS.

Previous studies on the uptake and incorporation of ^{35}S-labeled sulfate into carrageenan in *C. crispus* showed the labeled sulfate appearing as half-ester groups in both kappa- and lambda-carrageenan. In that study no attempt was made to select tissues of a specific nuclear phase in the life cycle. Recently, several investigators have reported that kappa-carrageenan is limited to gametophytic stages of the life cycle. The kappa/lambda ratio of whole fronds from female or male gametophytes that were gathered in the Woods Hole area was 1.3 while that of tetrasporic plants was 0.01, values which confirm observations reported by others. This finding prompted our present study, an investigation of sulfate incorporation into carrageenan fractions at each nuclear phase in both vegetative and reproductive tissues. Sulfation was greatest in marginal regions of the distal part of the plant. Plants lacking reproductive structures incorporated twice as much sulfate per unit fresh weight into carrageenan as those bearing such structures. An attempt was made to correlate the age of a plant as determined by number of dichotomies with incorporation of sulfate into carrageenan. To determine the relative distribution of kappa- and lambda-carrageenan throughout a plant, a female gametophyte bearing cystocarpic structures on its outer branches was divided at successive proximal branches into six samples. The kappa/lambda ratio in a sample consisting of distal margins with cystocarps was 0.2 while the region immediately proximal gave a value of 9.1. More proximal samples had ratios of 2.6 to 3.1. It would appear that the female gametophyte has highly localized depositions of kappa-carrageenan, especially in maturing regions of the stipe while the actively enlarging region at the margin and in the vicinity of cystocarp formation has less kappa-carrageenan relative to lambda-carrageenan.

This work was generously supported by grants GB-37149 from NSF and GM-12422 from NIH.

Acceleration, kinetic energy changes and gaits of locomotion in swimming bluefish. RICHARD S. FOX, GIOVANNI A. CAVAGNA AND ARTHUR B. DUBOIS.

We measured forward and lateral accelerations in swimming bluefish, and calculated velocity and kinetic energy changes therefrom. Forward accelerations up to 3.2 *g* occurred in bluefish when startled while swimming freely in a 10 by 15 foot pool. Four primary patterns or gaits of forward acceleration were seen. The fish swam in a one foot diameter tunnel inclined at 33° and containing water flowing from the pool. The first pattern consisted of a short burst of large forward accelerations sandwiched between several smaller but regular accelerations. The second consisted of two equal forward accelerations with each complete tail cycle. The third was two unequal forward accelerations with each cycle, indicating that the propulsion obtained by the fish driving his tail was greater to one side than to the other. In the fourth pattern, a single forward acceleration notched in one limb occurred with each tail cycle indicating that most of the forward propulsion was gained by driving the tail to one side. These patterns were recorded at speeds ranging from 0.4 to 3.5 mph. The fish swam with the second and third patterns at the low speeds less than 1 m/sec and tail frequencies less than 2.2 Hz. At higher speeds they preferred the fourth pattern. The kinetic energy change at 0.5 m/sec was about 0.0035 kg m, and at 1.5 m/sec it was about 0.023 kg m. These large changes in kinetic energy were associated with the single acceleration per tail cycle. This gait occurred at tail frequencies greater than 2.2 Hz. One possible explanation is that such a gait allows an optimum rate of muscle contraction, whereas another possibility is that the pattern of water flow at this frequency may give the tail better propulsion. The maximal acceleration of the fish when startled is approximately equal to that of a man jumping with maximal effort. Thus, fish would have enough strength to support their weight on the earth's surface.

The relationship between cell division and the ontogeny of the localizations which specify comb plates and photocytes in the Ctenophore Mnemiopsis. GARY FREEMAN.

The first differential division in Ctenophores during embryogenesis occurs at the 8-cell stage. The E macromere lineage that is formed will differentiate comb plates when it is isolated and the M macromere lineage will self differentiate to form photocytes, while the progenitor cell of these 2 blastomeres will form both cell types when it is isolated. The localizations of developmental potential which segregate at the third cleavage begin to be set up following the second cleavage.

Cytochalasin B (0.01 mg/ml) and 2–4 dinitrophenol (0.0005 M) reversibly block cleavage in *Mnemiopsis*. If the first cleavage is blocked the second cleavage will occur in these embryos at the same time that the third cleavage occurs in control embryos. The 4 blastomeres generated by the first two cleavages after the block show a normal 4-cell stage configuration; the subsequent pattern of cleavage is normal. Experiments with these blocked eggs in which nucleated fragments are formed that correspond to the region of the 2- or 4-cell stage blastomeres that would normally form the E and M macromeres show that the localizations of developmental potential at the 2- and 4-cell stages correspond to that of normal embryos at these stages.

If the second cleavage is blocked the next cleavage will occur at the time the third cleavage is taking place in control embryos; this cleavage is oblique; in most cases a 4-cell stage configuration of blastomeres results that corresponds to the 8-cell blastomere configuration with 2 end cells and 2 middle cells. Egg cutting experiments in which nucleated fragments of the blocked egg that would normally form either the E or the M macromere were made at different times showed that the localizations which specify comb plates and photocytes are set up at the time the control embryos cleave to form the 4-cell stage.

This work is supported by N.I.H. grant GM-20024-01.

The effect of thyroxine on the growth of the ascidians Perophora viridis *and* Amaroucium constellatum. MAKOTO FUKUMOTO.

There are several reports which indicate that thyroid hormones are found in ascidians. However, almost no attempt was made to establish the physiological significance of these hormones for ascidians.

It has been established that in *Perophora orientalis*, thyroxine and triiodothyronine increase the rate of vascular stolon growth and inhibit budding, while the goitrogen thiourea inhibits the elongation of the stolon and promotes the process of asexual reproduction.

In *Perophora viridis*, thyroxine also increases the rate of elongation of the isolated vascular stolon. This effect is observed at concentrations of 10^{-6} M and 10^{-5} M, but not at those of 10^{-7} M and 10^{-8} M. Asexual reproduction was only slightly inhibited. Triiodothyronine has a similar effect on the rate of elongation of the isolated vascular stolon. Triiodothyronine was more effective than thyroxine in that concentrations as low as 10^{-8} M had effect. The amount of stolon growth is proportional to the concentration of hormone. In a typical case in which 10^{-5} M thyroxine was used the stolon was 3 times the length of the control after 4 days of treatment.

Experiments on the oozooid of *Amaroucium constellatum* showed that thyroxine also had a growth promoting effect in this species. This effect was observed down to a concentration of 10^{-9} M. Thyroxine did not increase the growth of all parts of the oozooid to the same extent. The growth of the postabdomen was most accelerated compared with the thorax and abdomen.

Autoradiographic studies indicated that ^{125}I was bound by the endostyle, the tunic cells, the extracellular material of the tunic and the branchial basket of both species. Studies in which the iodine-125 containing material was extracted and chromatographed indicated that some of the iodine was found in thyroxine and triiodothyronine.

The author wishes to express his sincere thanks to Gary Freeman for his kind encouragement and discussion throughout this work.

Effect of calcium antagonism on the free amino acid regulation of cell volume in Modiolus demissus. STEPHEN J. GIRSCH AND SIDNEY K. PIERCE, JR.

When marine bivalves are challenged by a decrease in salinity, cell volume and osmolarity are regulated by the efflux of specific amino acids, chiefly taurine, glycine, alanine, and proline with their obligated water. Permeability of the amino acid effluxing sites is apparently synergistic, controlled by the external osmotic pressure and by the calcium ion concentration. The role of calcium in efflux control was examined by the substitution of other divalent ions for calcium, and by the application of calcium binding site antagonists.

Heart ventricles were suspended in aerated 1 ml organ baths with facility for rapid flushing and medium substitution. The release of free amino acids was followed by withdrawal of hematocrit tube aliquots from the bath, spotting on filter paper, spraying with ninhydrin solution, and drying at 70° C. Mechanical activity of the hearts was monitored by force transduction. Normally beating hearts in SW were flushed with isosmotic Ca^{++}-Mg^{++} free SW, allowed to become quiescent, and the heavy efflux of free amino acids monitored. The baths were next flooded with isosmotic SW's containing Ba^{++}, Co^{++}, Mn^{++} and Sr^{++} as the substitute divalent ions. Sr^{++} initiated a normal heartbeat, but was only partially effective at controlling amino acid efflux. Ba^{++} and Mn^{++} halted amino acid efflux, but were unable to initiate mechanical activity. Co^{++} had no effect.

The following calcium antagonists were applied at the 1 mM level in both SW and 50% SW: A23187(Eli Lilly), chlortetracycline, ruthenium red, terbium chloride, Verapamil(Knoll), and X537A(Hoffman-La Roche). Chlortetracycline was found to affect mechanical activity, but did not interfere with amino acid efflux. The latter three compounds caused mechanical alterations and were partially effective in blocking free amino acid efflux.

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Dependence of absorption on pH in rhabdoms of crayfish (Orconectes, Procambarus). Timothy H. Goldsmith.

Absorption of individual rhabdoms (photoreceptor organelles) was measured with a laterally-incident microbeam between 700 and 375 nm. The pH of the suspending medium was adjusted over the range ~2.0–9.7 using phosphate, citrate, and borate buffer systems. Difference spectra for total bleaches were measured either directly or by using typical baselines from other bleached rhabdoms.

The absorption maximum (λmax) of freshly isolated rhabdoms at neutral or slightly alkaline pH lies at about 535 nm. On the acid side of neutrality λmax also shifts to shorter wavelengths, but the effect is much larger. By pH 2–3 λmax is near 460 nm. This shift is completely reversible, even though the shape and (by inference) fine structure of the rhabdom are irreversibly altered by exposure to very acid pH.

The morphological integrity of the rhabdom can be preserved by exposure to glutaraldehyde, permitting more accurate absorption measurements. In the presence of 0.8–2.5% glutaraldehyde at neutral pH, absorption is similar to unfixed material, and a normal-appearing metarhodopsin with λmax at 512 nm forms when the organelles are irradiated with bright orange light. At pH >8.5 the metarhodopsin is readily photobleached, and absorption increases in the near ultraviolet. On the acid side of the scale, the absorption spectrum is not as profoundly altered as in unfixed material: exposure to pH 2 shifts the spectrum to 480–485 nm, rather than 462 nm. Moreover, the displacement is not completely reversible. Throughout the entire pH range, however, brief irradiation produces a metarhodopsin absorbing at shorter wavelengths than the rhodopsin. At pH 2–3 the metarhodopsin has λmax at 462 nm, and more prolonged irradiation leads to a final photoproduct with λmax at about 450 nm.

These observations are consistent with conformational changes in an opsin to which a retinaldehyde chromophore is attached as an aldimine.

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Neuromuscular analysis of closing in the cutter and crusher claws of the lobster. C. K. Govind and Fred Lang.

The closer muscle in the dimorphic cutter and crusher claws of the lobster, *Homarus americanus* receives two excitatory and an inhibitory axon. The cutter has been shown to have two distinct populations of short-sarcomere (fast) and long-sarcomere (slow) muscle fibers; one of the excitatory axons has been characterized as a fast axon (eliciting twitch contractions) while the second axon is assumed to be a slow one. The crusher has largely long sarcomere (slow) fibers; both excitatory axons are assumed to be slow, resulting in the slow tonic contractions of this claw.

We found that stimulation of the fast axon in intact, isolated cutter claws gave individual twitches which fatigue in about 45 sec at 2 Hz. The slow axon only responds at higher frequencies (10 Hz) with tonic contractions which are fatigue resistant. Surprisingly, the axons of the crusher also behaved as fast and slow, the former giving twitches and the latter only tonic contractions. The twitches were especially large when paired stimuli with a 2 msec interval between them were applied.

Intracellularly recorded excitatory postsynaptic potentials (EPSP's) in response to fast axon stimulation in the cutter gave large (4–15 mV at 1 Hz) poorly facilitating responses which fatigued after 90 sec at 10 Hz. Paired stimuli resulted in strongly facilitating EPSP's which often gave an active membrane response. The response to slow axon stimulation varied: the majority of impaled fibers gave EPSP's which were detected only at high frequencies of stimulation (20–30 Hz), however, some fibers gave large EPSP's (6–8 mV at 1 Hz). Most of the slow EPSP's facilitated strongly and were fatigue resistant.

The crusher, with its nearly uniform population of slow fibers, also gave fast and slow EPSP's similar to those in the cutter. One axon responded with large poorly facilitating EPSP's and fatigued rapidly. The second axon gave small highly facilitating EPSP's which were fatigue resistant. Thus the slowly contracting crusher rather enigmatically possesses a "fast" excitatory axon.

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Radio-labelling proteins of the sea urchin egg surface. James L. Grainger and Dennis Barrett.

Marchalonis and others have demonstrated that lactoperoxidase forms a complex with peroxide which can covalently bind iodide to tyrosyl residues of exposed proteins. Lactoperoxidase does not penetrate cell membranes, so that ^{125}I has been used selectively to label surface proteins. We have used the technique to probe the exterior of the sea urchin egg.

Arbacia eggs were dejellied by HCl-sea water, pH 5, and some treated with pronase until they could raise no fertilization envelopes. The 10^5 eggs were radioiodinated by exposure for 15 min at 25° C to 200 μCi $Na^{125}I$, with lactoperoxidase and H_2O_2 in 200 μl of sea water buffered with 50 mM phosphate at pH 7.3. About 5% of the label was bound under these conditions, without impairing fertilizability.

After thorough washing, eggs were fertilized, and independent of pronase treatment they lost on the order of one quarter of the label to the supernatant.

Embryos were fixed in ethanol at −20° C, gently homogenized in cold sea water, and fractionated on 2.5–4.0 M CsCl gradients. Fertilization envelopes and eggs were cleanly separated from each other and were found labelled to roughly similar extents. As expected, no counts were found in the region of the gradient characteristic of fertilization envelopes, if eggs had been pronase treated.

Further effort will be directed to the basis for the apparent porosity of the vitelline envelope to lactoperoxidase.

Supported by NIH grant GM 00265-15.

Environmental regulation of fish hemoglobin oxygen affinity: the role of ATP. Michael A. Granet, Martin B. Weiss and Dennis A. Powers.

During the past two years we have found that ATP is the allosteric modifier of boney fish hemoglobins. Our study describes some of the effects of ATP on *Fundulus heteroclitus* hemoglobins. Oxygen equilibria curves were done at two pH values, both with and without ATP. In both cases the ATP shifted the binding curves to the right (*i.e.*, depressed binding). In addition, both the Bohr effect and the subunit cooperativity were affected. When fish were acclimated to various temperatures, it was found that ATP pools were reduced at warmer temperatures (*i.e.*, 30°).

As the water temperature rises, the dissolved oxygen decreases. At higher temperatures, the fish appears to be able to bind more oxygen by raising the oxygen affinity *via* lowering ATP concentration. The depressed release of oxygen at the cells is overcome by lowering blood pH as the temperature rises, thereby enhancing the Bohr effect. This change in pH and ATP is finely tuned to optimizing the subunit cooperativity.

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A duplex flicker fusion curve recorded from the skate retina. DANIEL G. GREEN AND IRWIN M. SIEGEL.

Responses to flickering stimuli of equal light-dark duration were recorded from the skate retina using ERG b-waves, aspartate isolated receptor potentials, and intracellular responses from horizontal cells. Sufficient time was allowed at each level of intensity to ensure maximal flicker following. The graphs of critical flicker fusion (c.f.f.) against log I are double branched curves for the receptors, b-wave, and horizontal cells. The lower portion of the curve reaches an asymptote at 8 cps while the upper portion may follow as high as 30 cps. A sharp inflection point for the three kinds of electric activity is attained at a quantal absorption of 1000–2000 quanta/rod/sec. While this sort of duplex function is usually ascribed to rods and cones, the skate, on the basis of present anatomical and physiological evidence, is believed to have an all-rod retina. Since a small number of functioning cones might be responsible for the upper branch of the flicker fusion curve, we measured b-wave light and dark adapted spectral sensitivity and b-wave flicker intensity relationships using monochromatic stimuli. The results indicated that both light and dark adapted sunctions fit a Dartnall nomogram peaking at 500 nm. From the above, it seems reasonable to conclude that the duplex flicker curves derive from a rod which alters its response characteristics.

Intracellular recordings from S-units showed that two experimental conditions must be met before fast flicker responses can occur. The stimuli must be both intense enough to saturate the S-potential and prolonged in duration. If measurements were taken too soon after the onset of a moderately intense flickering stimulus, the retina failed to respond, resulting in a peaked c.f.f. *vs.* log I curve with maximal following at 5 cps. Thus it seems that the capacity to follow fast flicker is achieved by the photoreceptors in the process of recovering from illumination levels that usually produce rod saturation.

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Electrophoretic studies of dogfish lens proteins. GARY GRIESS AND SEYMOUR ZIGMAN.

Lens proteins have long been divided into water soluble and insoluble fractions. Microscopic examination of the insoluble precipitate reveals it to be composed of lens fiber cell "ghosts." The ghosts of dogfish (*Mustelus canis*) lenses are unusually high in protein and low in lipid content as compared to other cell membranes. They become completely soluble in 8 M urea solutions. Their protein composition was analyzed on 8 M urea acrylamide gels (Tris-Glycine pH 8.4). More definitive characterization was made by using a series of gel concentrations to determine the free electrophoretic mobility and gel retardation coefficient for each component. Eight major components of the water insoluble fraction were characterized while fifteen components were found for the soluble fraction. Comparison of mobilities and retardation coefficients in urea was made with lens crystallin fractions obtained by stepwise elution with phosphate buffer on DEAE-cellulose. Each of these fractions has several components which match the insoluble protein pattern.

Sulfonation of the lens proteins with Bailey's reagent improves resolution and confirms the identity of the insoluble fraction with lens crystallins. The subunit molecular weights calculated from the retardation coefficients are mostly in the range of 23,000–24,000 daltons which accounts for the single broad band obtained by SDS acrylamide gel electrophoresis.

The high concentration of protein associated with the fiber cell membranes imparts rigidity to the structure. Since lens proteins have a very low turnover, the incorporation of proteins from the lens plasma into the interface structure is a reasonable process.

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Corneal epithelial cell surfaces in elasmobranchs and teleosts as seen with the scanning electron microscope. C. V. HARDING, M. BAGCHI, A. WEINSIEDER AND V. PETERS.

The external surface of the mammalian cornea has been shown to consist of numerous fine ridges. These ridges are apparently responsible for holding the tear film, which provides an air-water interface that serves as the main refractive surface of the eye. Is the ridge structure also present on the surface of fish corneas, which are normally exposed to an aquatic environment? In the present investigation, a comparative study of the surface structure of fish corneas was made. Two species of elasmobranch were used (smooth dogfish, *Mustelus canis;* and the little skate, *Raja erinacea*); and 5 species of marine teleost (scup, *Stenotomus chrysops;* northern sea robin, *Prionotus carolinus;* summer flounder, *Paralichthys dentatus;* bluefish, *Pomatomus saltatrix;* toadfish, *Opsanus tau*). Freshly excised corneas were fixed in 2% buffered glutaraldehyde, post-fixed in 1% osmic acid, dehydrated with an alcohol or acetone series, dried in a critical point drying apparatus and coated with carbon and gold for examination with the scanning electron microscope. The results indicate that the two elasmobranchs (dogfish and skate) have a ridge structure similar to that in mammals, but have their own distinctive patterns. Preliminary observations indicate that the ridge pattern in a freshwater teleost (goldfish) is similar to the elasmobranch pattern. The marine teleosts also have a ridge structure; however, they have a pattern distinctly different from that in the elasmobranchs. Ridges, 0.2 micra in width, and many micra in length, form more intricate patterns than occur in the dogfish and skate. In all cases, the patterns appear to have species specificity. There is evidence that these ridge patterns are responsible in the marine teleosts for binding an external coating material which constitutes the external surface of the eye. The amount of coating material varies from cell to cell, and is sharply delineated at cell borders.

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Transport of L-leucine by toadfish liver in vivo. Audrey E. V. Haschemeyer and Alan P. Hudson.

A new method has been developed for the characterization of amino acid transport systems in liver based upon the rapid uptake of pulse-injected ^{14}C-amino acids supplied via the hepatic portal vein relative to ^{3}H-mannitol used as a marker for extracellular space. The uptake of L-amino acids into intracellular space reaches equilibrium in 1 min at 20°. At a tracer dose of 2 nmole L-leucine in 0.1 ml (0.02 mM), fractional uptake A_1 is 0.46 ± 0.04 (17) or 0.9 nmole actual uptake per average 7-g liver. Saturation of uptake begins to appear at injection concentrations of 1 mM. At 15 mM fractional uptake is greatly reduced: $A_1 = 0.17 \pm 0.06$ (11). Actual uptake is 250 nmole for the 0.1 ml injection; doubling the dose increases uptake to 350 nmole. Extrapolation of a double reciprocal plot of uptake *vs.* dose indicated a theoretical maximal uptake of 670 nmole at infinite concentration in a 0.1 ml pulse. This amounts to about half of the normal free leucine content of toadfish liver (1.4 μmole/7-g liver), and is adequate relative to leucine utilization in protein synthesis (100 nmole/min/7-g liver) in 20°-acclimated fish at 20°.

The apparent accumulation against a concentration gradient at low doses and the saturation of uptake at high doses are indicative of an active transport process for L-leucine in liver. Possible transport of other L-amino acids by the leucine system was tested by measuring their effect at 15 mM concentration on ^{14}C-L-leucine uptake. Inhibition comparable to that found for 15 mM L-leucine (about 60%) was obtained with isoleucine and phenylalanine. No effect was observed with alanine, glycine, histidine, lysine and proline. Variable effects (15–40%) were obtained with methionine, threonine, aspartic acid, cysteine, valine and tyrosine. The transport system resembles the L system described by Oxender and Christensen in Ehrlich ascites cells.

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Autoradiographic localization of sulfated macromolecules during development of Arbacia punctulata*; normal and sulfate deficient media.* Mark N. Hill, Paul A. Rosenberg and Robin A. Wallace.

It has been suggested that sulfation of acid mucopolysaccharides is involved in normal morphogenetic movements during gastrulation in the sea urchin embryo, and that these macromolecules play a determinative role in selective contacts and adhesions of filopodial extensions of mesenchymal cells. Unlike the European and Japanese sea urchins, *Arbacia* embryos proceed through gastrulation in the absence of sulfate. They do not, however, form the oral contact through the ventral shift of the archenteron to the stomadeum, mediated by mesenchyme.

Rate of sulfate uptake was determined by incubating aliquots of 25,000 embryos through development at 22.5° C for 0–40 min in $^{35}SO_4$ (4 μCi/ml MBL sea water containing half normal sulfate). The embryos were quickly washed in 0.22 M sucrose in MBL sea water and assayed for total label. Maximum rates of uptake occurred 2–3 hr post-hatching (11.5–12.5 hr post-fertilization) and at 16–18 hr p.f. when the prism stage is emerging.

For autoradiographic studies, embryos at 11 hr p.f. (blastula) and 16 hr p.f. (mid-gastrula) were treated with 5.0 μCi $^{35}SO_4$ per ml sulfate deficient sea water for 60 min and quickly washed in 0.22 M sucrose in MBL sea water. The former was transferred to sulfate deficient sea water and the latter to MBL sea water for further development. The embryos were collected at 1.5 hr intervals and fixed with Motomura's fixative No. 5 for 24 hr. Sections of 5–6 μ were made by paraffin method. Temperature was kept at 22° C throughout the experiment. Dipping method was used for autoradiography with Kodak NTB2 emulsion, the exposure time being 5 days. The autoradiographic slides were counterstained with alcian blue and neutral red.

Autoradiographic grains in normal embryos at 17 hr p.f. were localized at the archenteron tip and on the inner ectodermal surface of the acron; at 23 hr p.f. the sites of maximum incorporation were the tip of the ventrally-shifted archenteron and the inner surface of the invaginating ectoderm (stomadeum). In sulfate-deprived embryos at 16 hr p.f. grains covered the archenteron surface; at the stage of arrest (21 hr p.f., complete gastrula prior to ventral shift) grains localized at the archenteron tip but not on the inner ectodermal surface. Alcian blue stain coincided with grains when present.

These studies show that incorporated sulfate is involved in the interaction of the archenteron endomesoderm with the acronal ectoderm and subsequently with the ectoderm of the stomadeum. The sulfate is most likely incorporated into mucopolysaccharides. Furthermore, there appears to be a species difference in the utilization of endogenous sulfate for gastrulation.

This study was performed in the Fertilization and Gamete Physiology Training Program (NIH 5-T01-HD00026-12) and the Embryology Course (NICHD) at the Marine Biological Laboratory.

Accessibility of the chromophoric site of squid retinochrome. RUTH HUBBARD AND LINDA SPERLING.

Retinochrome is a photosensitive pigment that occurs in cytoplasmic membranes in the visual cells of cephalopods. Like rhodopsin, retinochrome has λ_{max} near 500 nm, but its chromophore is all-*trans* retinal, which light isomerizes to 11-*cis*, the reverse of the situation in rhodopsin. In both pigments the aldehyde group of retinal forms an aldimine (Schiff base) with an amino group on the opsin. The rhodopsin chromophore is not accessible until it is photoisomerized or the rhodopsin is denatured. The chromophore of retinochrome is more reactive. (1) Hydroxylamine converts retinochrome in the dark to all-*trans* retinal oxime (λ_{max} 367 nm) plus opsin. (2) Sodium borohydride reduces it to retinyl-N-opsin (λ_{max} 335 nm). (3) λ_{max} of retinochrome shifts from 500 to 515 nm as the pH is raised from 6 to 10, with a loss of absorption above pH 8; meanwhile above this pH a second band appears with λ_{max} 375 nm. These changes are reversible. The equilibrium between the long and short wavelength forms probably involves the titration of the aldimine bond: retinyl—C=N$^+$(H)·opsin (λ_{max} 500–515) $\rightleftharpoons$ retinyl—C=N·opsin (λ_{max} 375) + H$^+$. (4) If retinochrome is incubated with all-*trans* 3-dehydroretinal (retinal$_2$) in the dark, some 3-dehydroretinochrome (retinochrome$_2$, λ_{max} about 520 nm) is formed. Conversely, when retinochrome$_2$, made by adding all-*trans* retinal$_2$ to retinochrome opsin, is incubated with all-*trans* retinal, some retinochrome is formed. Retinal and retinal$_2$ therefore can replace each other as chromophores in the dark: retinochrome + all-*trans* retinal$_2$ $\rightleftharpoons$ retinochrome$_2$ + all-*trans* retinal.

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Further studies of particle release from activated sea urchin eggs. SADAYUKI INOUE, ERNEST COUCH, MARKHAM KIRSTEN AND ALBERT GROSSMAN.

Sea water, in which *Arbacia punctulata* eggs are fertilized, has been found to contain structures about 300 Å in diameter. Using the "platinum shadowing-replica film" technique for electron microscopic study, these particles were readily observed in the surrounding sea water within 10 min after fertilization, but were relatively sparse in sea water containing equivalent numbers of unfertilized eggs. It is unlikely that these particles originated from the jelly coat of the egg since pre-treatment with pH 5 sea water (which removes the jelly coat) did not prevent the appearance of these particles subsequent to fertilization. Parthenogenic activation of eggs by hypertonic sea water also caused release of these particles into the surrounding medium. Approximately 50% of the eggs so treated demonstrated fertilization membranes. In all cases eggs were initially washed with about 20 volumes of Millipored sea water (pore size 0.45 μ) and then fertilized or parthenogenically activated in similarly treated sea water. Electron microscopic examination of such filtered sea water demonstrated little artifactual material when examined by platinum shadowing or by negative staining with uranyl acetate (0.3%). With the latter technique, some particulate matter was observed in sea water containing unfertilized eggs. Although we are uncertain of the origin of these structures at present, it is possible that such particles are released from sea urchin eggs in response to their contact with sea water.

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Isolation and growth of a cold and calcium labile spindle from Chaetopterus *oocytes in solutions containing pig brain tubulin.* SHINYA INOUÉ, GARY G. BORISY AND DANIEL P. KIEHART.

Purified tubulin solutions stabilized and augmented the birefringence (BR) of isolated *Chaetopterus* spindles. Tubulin was extracted from pig brain tissue in cold PEG buffer (0.1 M PIPES, 1 mM EGTA, 2.5 mM GTP, pH 6.94 at 25° C) and purified by two cycles of a reversible, temperature-dependent assembly-disassembly procedure. Metaphase arrested oocytes of *Chaetopterus* perfused with PEG buffer without tubulin lose their spindle BR at 1.5 nm/min. In this hypotonic, calcium chelating solution the cell lysed within 1.5 minutes, and after a brief transient rise, the BR disappeared in *ca.* 3.5 minutes from the time of buffer application. Cells perfused with tubulin in PEG buffer also showed BR decay at the same rate until cell lysis. Immediately upon cell lysis the spindle BR increased, initially at *ca.* 2.3 nm/min and then more slowly until BR approached or exceeded intact cell values. Spindle and asters grew considerably larger than those in intact cells. From the kinetics of the transient BR increase after lysis, we infer that initially *Chaetopterus* cytoplasmic and added pig tubulin contribute to increased BR; further increase appears to arise from added pig brain tubulin alone. Isolated, growing spindles were depolymerized rapidly at 6° C; upon return to 23° C, spindle BR returned slowly in tubulin-PEG. The BR of the isolates also decayed in solutions containing calcium ions 1 to 2 mM in excess of EGTA. The isolates however did not respond, or responded very slowly, to 1 mM colchicine or Colcemid and to dilution of tubulin of PEG buffer. Microinjection into *Chaetopterus* oocytes of tubulin-PEG, but not PEG alone, enhanced spindle and aster BR which reversibly disappeared upon chilling the cell.

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"Muscle" proteins in the electric organ of the Torpedo. BENJAMIN KAMINER AND ESZTER SZONYI.

The electric organ of *Torpedo* develops from a myoblast, but contains no features resembling muscle as do some weak electric organs. We nevertheless considered that this strong electric organ might contain "muscle" proteins modified in their structural organization and function. We reported previously on the isolation and characterization of tropomyosin from this organ and now report on the isolation of actomyosin. We extracted actomyosin in Weber-Edsall solution after blending and washing the tissue at low ionic strength. Parallel extractions were made from *Torpedo* muscle. On SDS gel electrophoresis myosin (heavy and light chains), actin and tropomyosin were identified. The ATPase activity (in 2.5 mM $MgCl_2$, 25 mM Tris-buffer, pH 7.6) was about P_i, 0.15 μmoles/mg protein/min, was relatively unstable and about 20–25 × less than that of the muscle actomyosin. The ATPase was activated by $MgCl_2$ and $CaCl_2$, a maximum being obtained at a conc. of about 2.5 mM. EGTA (1 mM) reduced the ATPase by 24–36% thus showing some Ca dependence. ATP reduced the relative viscosity of the actomyosin (in 0.6 M KCl) by 10% as compared to 56% in the case of the muscle actomyosin. On dissociating the myosin from actin with ATP, the actin pellet was not clear and in SDS gels, myosin and tropomyosin were present besides the major actin band. Likewise, the myosin fraction contained impurities of actin and other unidentified proteins. The yield of actomyosin was low. The ratio of actomyosin to tropomyosin from the electric organ was 0.7:1 which is markedly less than the ratio of 15:1 from the muscle and 18:1 from rabbit muscle. This relative abundance of tropomyosin suggests that the "muscle" proteins are assuming modified functions in the electric organ.

This work is supported by a grant from N.S.F.

Limulus *lateral eye: intracellular recordings* in situ *and in organ culture.* E. KAPLAN, D. S. BAYER AND R. B. BARLOW, JR.

Single ommatidia in the unexcised lateral eye of *Limulus* fire impulses in response to very few photons and encode light intensity over a range of approximately ten log units. In an attempt to understand the mechanisms underlying these properties, we recorded intracellularly from retinular and eccentric cells *in situ*. Our main findings are: (1) In the dark adapted eye, the membrane potential of the visual cells undergoes small spontaneous fluctuations of about eight mV in amplitude and large fluctuations of up to 80 mV in amplitude. The large potential fluctuations (LPFs) appear to be regenerative. (2) Dim illumination increases the frequency of the LPFs but not their amplitude. However, they are usually large enough to elicit impulses in the optic nerve fibers, and may provide a mechanism for detecting weak stimuli. (3) The intensity function of the receptor potential of the retinular cell is similar in shape and in range to the intensity function of the impulse discharge from the eccentric cell. This suggests that the large operating range of the unexcised eye originates in the retinular cells themselves.

To avoid the problem of animal movement, we recorded from slices of the lateral eye in organ culture. For periods of at least three days the visual cells in organ culture retain the properties stated above for the *in situ* preparation. Control experiments in sea water usually produced results typical of the excised eye; however, on occasion intact-like responses were recorded for a few hours after excision. Such responses could be recorded from receptors located only on the cut edge of the retinal slice. Apparently, the retinal tissue restricts the diffusion of substances to and from the receptors and thereby leads to a decrease in the sensitivity of the eye.

E. Kaplan was a Fellow of the Grass Foundation during the summer of 1973.

Observations of luminescent bacteria in continuous culture. LYNDA A. KIEFER, HOLGER JANNASCH AND KENNETH H. NEALSON.

Eberhard, in 1972, demonstrated the presence of a small molecule produced by marine luminous bacteria which is responsible for the induction of the luminescent system. Autoinduction, in batch culture, occurs only after the cell density has reached a characteristic concentration during mid-exponential growth and the luminescent activity subsequently ceases during stationary phase.

Free-living luminescent bacteria have never been observed to luminesce in the open ocean and Nealson has postulated that the absence of luminescent activity is a result of an insufficient concentration of inducer substance in the environment to promote the activation of the luminescent system.

In the present investigation, this model has been examined using the continuous culture technique. Continuous culture, unlike batch culture, permits the maintenance of constant populations in an externally controlled chemical environment over long periods of time.

Photobacterium fisheri, strain 121, was cultured in a chemostat apparatus in which the population density was determined by limiting the substrate (glycerol) concentration and maintaining a constant flow rate. Light production was shown to be sustainable over several days when a cell density greater than the induction density (determined in batch culture studies) was maintained, thus demonstrating a potential for continuous light emission for long periods.

After steady-state conditions were achieved at high cell density, dilutions of the limiting substrate by factors of two resulted in proportional and predictable decreases in cell density.

Light emission, on the other hand, was proportional to dilution only until the cell density of induction was reached. Here, light emission was rapidly extinguished while the cell density remained at the predicted value, thus supporting the critical concentration model for inducer activity.

TAME hydrolase activity and activation of sea urchin eggs. MARKHAM KIRSTEN, ERNEST COUCH AND ALBERT GROSSMAN.

Within minutes after fertilization of *Arbacia punctulata* eggs, there is release of tosylarginine methylester (TAME) hydrolase activity into the surrounding sea water. Such activity is also found in sea water after parthenogenic activation of sea urchin eggs by hypertonic sea water (6 g NaCl added per 100 ml sea water). Approximately 50% of the eggs exposed to this treatment developed fertilization membranes. An equivalent number of eggs that were fertilized by sperm (more than 90% developed fertilization membranes) released a proportionately greater quantity of TAME hydrolase into the surrounding sea water. Passage of sea water obtained from sperm or parthenogenically activated preparations through Millipore filters caused loss of TAME hydrolase activity. A solution of trypsin made up in sea water to contain activity equivalent to that found in the preparations cited above, about 0.1 μg/ml, was relatively unaffected by Millipore filtration. This observation tends to support our previously held notion that TAME hydrolase is not released into sea water in a free form. In an accompanying report evidence is presented which indicates that the enzyme is particle-bound.

The TAME hydrolase released into sea water was found to be inhibited by 1 mM concentrations of Zn^{++}, Cu^{++}, and Fe^{+++} ions, and relatively unaffected by 1 mM Pb^{++}, Ca^{++}, Mg^{++}, and N-ethylmaleimide.

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Eye withdrawal reflex evoked by dorsal root stimulation: electrotonic coupling and somatic impulse initiation in fish oculomotor neurons. H. KORN AND M. V. L. BENNETT.

Mechanical stimulation of head and anterior trunk can induce withdrawal of both eyes in the Puffer fish (*Spheroides*). Efferent nerve volleys or EMG's of medial and lateral rectus muscles of both eyes indicate that stimulation of anterior dorsal roots or spinal cord evokes simultaneous contraction of these muscles. Underlying synaptic events were studied by intracellular recordings from medial rectus motoneurons in both curarized and uncurarized fish. Spikes evoked by dorsal root stimulation arise from large and prolonged EPSPs (latency: 7–10 msec) with voltage threshold close to that for direct stimulation. These spikes are blocked by small hyperpolarizing currents, indicating that they are initiated in or near the cell soma, presumably through activation of the dorsal column and reticular afferent pathways. After spinal cord stimulation, this somatic EPSP is consistently preceded by one or two spikes (latency: 1–2.5 msec) arising abruptly from the baseline. Large hyperpolarizing currents delay these spikes, but little EPSP is seen to underly them. These impulses are initiated in dendrites apparently by activation of ascending collaterals of antidromically activated vestibulo-spinal neurons. Similar abruptly rising impulses are evoked by stimulation of the ipsilateral horizontal semicircular canal. Cell bodies of oculomotor neurons are electrotonically coupled to each other and antidromic stimulation induces graded depolarizations due to spread of activity from neighboring neurons. These depolarizations can summate with somatic EPSPs to excite the cells. The synaptic interrelations are such that electrotonic coupling tends to synchronize neuronal activity during the eye withdrawal reflex.

Acoustic telemetry of electrical activity from brain of free-swimming dogfish. NAIPHINICH KOTCHABHAKDI, JOHN KANWISHER AND C. L. PROSSER.

Unit activity was recorded by metal microelectrodes from cerebellum and optic tectum of *Mustelus canis*. Amplified signals were telemetered as frequency modulation of a 80 Khz acoustic transmitter attached to the fish. The original signals were recovered by a phase-locked receiver. Acoustic difficulties due to standing waves, absorption by micro-bubbles and doppler effects during swimming were overcome by recording in a large pen and by appropriate receiver circuitry. Rhythmical tonic spontaneous activity of cerebellar units was interrupted by quiescent periods. Some units discharged in correlation with flexion of the tail during swimming. Others responded to mechanical stimulation of tail and body. Units in optic tectum were sensitive to moving objects, including other fish, in the visual field. Variability of neural activity was noted which was not directly correlated with observed external stimuli. Simultaneous recordings of electrocardiograms and breathing electromyograms were also obtained. The technique of acoustic telemetry may be useful for relating neural activity to behavior of free-swimming fish.

This work was supported by NSF GA 3540X and Rockefeller Foundation.

Demonstration of lobster CNS tissue in monolayer cell culture. E. A. KRAVITZ, B. R. TALAMO, R. M. GROSSFELD AND K. D. EPSTEIN.

Cells have been isolated from the ventral nerve cord of first through fifth stage lobster larvae and placed in collagen coated dishes in monolayer cell culture. Conventional mammalian cell culture techniques are used throughout the experimental procedure. Standard cell culture media are used for dissection, trypsinization and plating cells (L-15 medium, fetal calf serum, glutamine and antibiotics) with salts added to bring the tonicity to that of lobster hemolymph. Morphological observations and time-lapse cinephotography demonstrate that cells of varying sizes (up to 50 μ) extend processes at a rate of approximately 0.1 mm/day.

The specificity of marine sponge aggregation factor. WILLIAM J. KUHNS AND MAX BURGER.

Rabbit antisera were prepared against purified aggregation factors (AF) derived from three species of marine sponges (1) *Microciona prolifera,* (2) *Haliclona occulata,* (3) *Halichondria panicea.* Sera were characterized by (a) the presence of specific precipitins and (b) biological effects as indicated by their ability to inhibit AF activity when preincubated with AF prior to the addition of the corresponding chemically dissociated cells. Precipitating activity and anti-AF activity appeared to parallel each other as judged by (1) the absence of precipitation when supernatants from mechanically dissociated cells were mixed with anti-AF and (2) the occurrence of precipitation when supernatants from chemically dissociated cells were mixed with anti-AF. Species specificity of each of the three antisera against mechanically dissociated cells of the homotypic species was demonstrated by exposure of the corresponding cells and of heterotypic cells to antibody followed by immunofluorescent staining with fluorescent labelled goat anti-rabbit serum. However, partial cross reactions between chemically dissociated *Microciona* cells and anti-*Haliclona* serum could be shown. We found a similar partial reaction between these two species when titrating the factor on heterotypic cells. Experiments were carried out which indicated that univalent antibody (F_{ab}) fragments and whole antiserum from which they were derived could specifically inhibit the re-aggregation of mechanically dissociated cells. We conclude therefore that purified AF is directly involved in initiating specific aggregation and that the specificity in two species investigated may be only quantitative rather than qualitative.

Can the firefly see colors? ABNER B. LALL AND TOM JENSEN.

Electrical responses (ERGs) elicited by light flashes (0.4 sec) of various wavelengths (320–700 nm) were recorded from the compound eyes in two species of fireflies, *Photuris versicolor* and *Photinus pyralis,* under conditions of dark- and chromatic light-adaptation. Spectral mechanisms were studied by obtaining spectral sensitivity curves measured in terms of log relative numbers of photons per flash at various wavelengths needed to evoke a constant amplitude (300 μV to 10 mV) of the electroretinogram. The spectral sensitivity curves from the dorsal region of the compound eye of *Photuris versicolor* fell into two groups, one with a single maximum at about 550 nm, and the second with two maxima, one at about 550 nm and the other at about 360–380 nm. The spectral sensitivity curves obtained from both the dorsal and the ventral regions of the compound eyes in *Photinus pyralis* possess two regions of maximal sensitivity. One group of curves had maxima in the near-uv (360–380 nm) and in the yellow (565 nm) regions of the spectrum, while a second group were maximally sensitive in the blue-violet (400–450 nm) and also in the yellow (565 nm). A very striking finding was the presence of a deep depression of sensitivity in the region 500–525 nm followed by a sharp increase in sensitivity, 1.5 to 2.5 log units in less than 50 nm. The dark-adapted spectral sensitivity curves in the yellow region of the spectrum showed a close correspondence with the bioluminescence emission spectrum of *Photinus pyralis*. By appropriate chromatic adaptation, one of the two peaks, either the yellow or the near-uv (or the blue-violet), could be selectively depressed in both species. The physiological evidence presented here for the presence of (a) two spectral mechanisms, near-uv and yellow, in the dorsal region of the compound eye of *Photuris versicolor,* and (b) three spectral mechanisms, near-uv, blue-violet and yellow, in *Photinus pyralis* compound eye, suggests that the fireflies probably have color vision.

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Thallium fluxes in the squid giant axon. DAVID LANDOWNE.

In many systems thallous ions are equivalent to potassium ions. The two ions have almost the same crystal and hydrated radii. I have compared their movements across the membrane of the squid giant axon by the use of radioactive tracers. The resting influx of Tl^{204} is two to three times larger than K^{42}. Ouabain reduces the resting influx of thallium by about one half, thus thallium is pumped by the sodium pump. More Tl^{204} is pumped than K^{42} suggesting that the pumping site has a higher affinity for thallium. As this has already been shown for the activation site, this suggests that the two sites may be the same. The ratio of the influx remaining in ouabain to the efflux is about 20 which is consistent with a passive distribution across a membrane with a 75 mV resting potential.

The fraction of Tl lost from the axon per nerve impulse is the same as for K. The extra influx per impulse is also the same for the two ions when measured from sea water with ouabain. From sea water without ouabain the extra K influx is about 30% higher and the extra Tl influx, 100% higher than in ouabain. This suggests that the passage of nerve impulses promotes enhanced pumping and, as at rest, the pump preferentially accumulates Tl. The ratio of the ouabain sensitive portions of the extra influx is not significantly different from the ratio of the ouabain sensitive portions of the resting influx.

This work was supported by a grant from the National Science Foundation (GB 36859).

In situ *experimental manipulation of diatom assemblages from salt marsh* Aufwuchs *communities.* J. J. LEE, J. HAGEN, C. A. MASTROAOLO AND H. RUBIN.

An experimental apparatus for the incubation of isolated natural *Aufwuchs* communities under close to ambient conditions has been developed. The apparatus retains all organisms larger than 1 μm, permits free exchange of culture fluids with the surrounding sea water, and is rugged enough to withstand the stresses of prolonged incubation in the field and severe storms. Experimental vessels constructed from polystyrene tissue culture flasks are perforated on the bottom with 12 1.25 cm holes to which are bonded nylon filters (Millipore NRWP 142–50). The experimental flasks are held in a Lucite rack designed to hold each flask horizontally 3 cm from the substrate. The racks are designed so that a 1 cm high chamber is below each flask. The floor of each chamber is perforated with many 0.6 cm holes. The apparatus is suspended above the substrate and is anchored between poles driven into the marsh. The apparatus seems ideal for use in *in situ* experiments on the effects of grazing by and competition among microherbivores.

In 2 separate experiments the apparatus was used in Sippewissett Marsh, Massachusetts, Jamaica Bay and Southampton, New York. In the first, populations from each field location were transplanted to each of the other locations. A mixture of the inocula was also incubated. In the second experiment the community at each location was incubated with reagent grade lumps of Fe, Cu, Zn, Cr, Pb, and with crude oil. All experiments were in triplicate. Initial results from the Sippewissett Marsh experiments show both quantitative and qualitative changes in the diatom population structure. After the 2nd week, Fe depressed the total growth of the assemblage to ~30–50%; Cu or Pb ~5–45%; Zn ~15–100%; Cr ~15–70%; and crude oil ~5–50% of controls. Population structure studies are proceeding slowly and should be completed in 2 years. Initial results are promising: *i.e.,* in the first week of incubation at Sippewissett the presence of crude oil completely inhibited the growth of *Melosira nummuloides* but enhanced the growth of *M. sulcata;* growth of *Synedra fasiculata* was relatively enhanced by the presence of Fe, Cu, Zn and Cr; *Opephora martyi* was strongly depressed by Cu; Zn more than doubled the proportion of *A. hauckiana* in the population, and all ions depressed the growth of *Nitzschia microcephala.*

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Isolation and characterization of cell walls from developing Fucus *embryos.* A. C. Ley and R. S. Quatrano.

Eggs of *Fucus vesiculosis* L. can be shown by electron microscopy to have no cell wall. Fifteen minutes after fertilization a carbohydrate cell wall, observable by plasmolysis, staining, and birefringence, surrounds the embryo. Cell wall ghosts (CWG) were prepared by homogenizing zygotes in distilled water, collecting the walls by centrifugation and repeating the homogenization several times in distilled water, ethanol:0.1 N NaOH (3:1), and 0.5% Triton-X-100. CWG so prepared retained the characteristic morphology of the zygote and the *in situ* staining and birefringent properties. Addition of radioactive sugars and native polysaccharides to the initial homogenate demonstrated less than 0.5% contamination of CWG by the cytoplasm. The carbohydrate composition of CWG at different times after fertilization was determined by extraction of the major polysaccharides and use of specific colorometric reactions to measure the amount of each. At twenty minutes, CWG are 60% by weight uronic acid polymers (alginic acid). The remaining 40% consists of a KOH insoluble glucan (cellulose) and trace amounts of fucose containing polymers (fucoidan). However, CWG isolated at six hours were 20% by weight fucoidan. Zygotes incubated from 0–6 hours after fertilization in a 50% sea water solution (development was normal) containing ^{3}H-fucose incorporated the sugar into the fucoidan fraction of CWG isolated at six hours. This may indicate that, at least in part, the fucoidan incorporated into cell walls of developing *Fucus* zygotes is synthesized *de novo.* The remainder of the cell wall at six hours is cellulose (20%) and alginic acid (60%). CWG isolated at later times show no significant changes in these relative amounts. The incorporation of these polysaccharides, which are either preformed during oogenesis or synthesized after fertilization, into a completely new cell wall deposited synchronously in a single-cell population may serve as a model system to study mechanisms and regulation of cell wall biogenesis.

We thank Dr. M. Gibbs for the suggestion to reduce the osmotic strength of sea water in order to enhance the uptake of labeled sugars. This work was supported by grants from the NSF (GB-37149) and PHS (GM-19247).

Effects of internal calcium on ionic currents in perfused squid giant axons. Carl Lynch and Ted Begenisich.

Squid giant axons were perfused and studied under voltage clamp in ASW. The isotonic perfusion solutions were HEPES buffered (pH 7.3) 275 mM K, 44 mM Na, sucrose, and the anions used were variously isethionate, glutamate or chloride. Control and test solutions contained 2 mM EGTA and Ca (1 mM, 5 mM, 10 mM), respectively. The axons tolerated the internal Ca as well as the isethionate, glutamate or chloride control solutions alone, and the leak current did not increase substantially in the presence of Ca *versus* control. In these normal ionic strength solutions, the sodium currents were only slightly if at all reduced. The curve of peak sodium conductance *versus* membrane potential was shifted only slightly (4 mV) if at all and in the direction of more positive internal potential. This shift is opposite the direction expected if Ca ions are neutralizing the negative surface charge on the inside of the membrane. There did appear to be some reduction (10%) of sodium currents; however, this was not a consistent finding.

Experiments were also performed at low ionic strength using buffered 44 mM Na glutamate and sucrose. Maximum sodium conductance was reduced by 46 and 48%, for 5 and 10 mM Ca, respectively, the reduction in current being only slightly reversible.

Experiments at normal ionic strength were performed with and without series resistance compensation with no alteration in results. Ca activity was measured with a divalent cation electrode, and it was found the glutamate complexed only 40% of the Ca and isethionate complexed 10% of the Ca in the solutions used.

Respiratory adaptability in relation to vertical zonation in littoral and sublittoral snails. ROBERT F. MCMAHON AND W. D. RUSSELL-HUNTER.

Six species of subtidal and intertidal snails were collected from Nobska Point and Manomet Point, Massachusetts. At Nobska and Manomet the subtidal snails, *Lacuna vincta, Mitrella lunata* and *Acmaea testudinalis,* and the intertidal snails *Littorina obtusata, L. littorea* and *L. saxatilis* were collected. Oxygen uptake rates in sea water for selected size groups from each collection were recorded at 5° C intervals from 5° to 45° C with Clark-type polarographic oxygen electrodes. The three sublittoral species showed a similar respiratory pattern with uptake increasing at a constant rate until 25° or 30° C followed by a steep decline to thermal death at 35° C.

As often noted, the three temperate Atlantic species of *Littorina* demonstrate a clear vertical zonation in their littoral distribution: *L. obtusata* near low water of neap tides; *L. littorea* as the dominant snail of the midlittoral; and *L. saxatilis* living around the high water level of neap tides. Other physiological adaptations to level have been claimed for these three species (*e.g.*, in reproduction and desiccation resistance).

Oxygen consumption in *L. obtusata* rises to 25–30° C and then declines slowly thereafter until thermal death at 41° C. *L. littorea* shows an increase in uptake rate to 20° C followed by either little or no further increase in rate until 30–35° C after which the rate decreases until thermal death ensues at 43° C. *L. saxatilis* (found in the high intertidal region) shows increase in uptake rate of oxygen until 20–25° C and then a sharp decline until 30° to 35° C followed by a low and apparently stable rate of uptake until thermal death at 45° C. Therefore, the three congeneric intertidal species are different in their respiratory adaptations and these are related to vertical position. *L. obtusata* shows a greater tolerance of high temperatures than sublittoral species. Midlevel *L. littorea* has an ability to maintain a stable respiratory rate over a wide range of environmental temperatures (20°–35° C). The highest intertidal form, *L. saxatilis,* survives high environmental temperatures (35–45° C) by reduction of O_2 uptake in a form of a reversible torpor.

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Independence of maternal mRNA activation from polyadenylation in Arbacia. ANTHONY MESCHER AND TOM HUMPHREYS.

A sequence of polyadenylic acid occurs at the 3′-OH end of most messenger RNA molecules in eukaryotes and is thought to play some role in the metabolism of such RNA. Following fertilization of sea urchin eggs, when protein synthesis on maternal mRNA is increasing rapidly, measurements of poly-A by hybridization of embryonic RNA with ^{3}H-polyuridylic acid show that the amount of poly-A doubles within 90 minutes. This increase involves the adenylation of cytoplasmic RNA and is essentially complete by the two-cell stage in *Lytechinus pictus* (Slater *et al.*, 1972, *Nature,* **240**: 333). Using the same basic assay procedure, we have determined the content of poly-A in eggs and early embryos of *Arbacia punctulata* and confirmed the results obtained with *Lytechinus.* We have investigated the relationship between poly-A content and the activation of protein synthesis using 3′-deoxyadenosine to inhibit poly-A synthesis. Polyadenylation is suppressed when eggs are preincubated in artificial sea water containing 3′-deoxyadenosine, inseminated and allowed to develop in the drug for two hours. At a concentration of 200 μg/ml, 3′-deoxyadenosine blocks the increase in poly-A content by 67% and at 750 μg/ml it inhibits 96%. Such massive concentrations of nucleoside might be expected to have a general toxic effect. However, adenosine at 1 mg/ml gives no inhibition of polyadenylation and embryos growing in high levels of 3′-deoxyadenosine, although delayed by about one hour by late cleavage stages, do not arrest until blastula stage.

Protein synthesis, measured as the percentage of radioactive leucine entering the cell which is incorporated into protein during a 5 minute pulse, was not significantly altered by 3′-deoxyadenosine. Control embryos incorporated 31.3 ± 2.9%, while those in 3′-deoxyadenosine at 200 μg/ml and 750 μg/ml showed 31.9 ± 1.3% and 25.9 ± 2.8% incorporation respectively. That the adenosine analog had little or no effect on protein synthesis suggested that poly-A formation was not necessary for activation of mRNA. This possibility was tested more directly by comparing the polysomes from normal and treated embryos. No diminution in size could be detected in polysomes from those embryos in which polyadenylation had been inhibited. The number of polysomes appeared to be approximately the same also, since incorporation of ^{3}H-leucine into nascent protein on polysomes was 8.6% of the TCA-soluble pool in controls and 9.1% and 7.4% in embryos grown in 3′-deoxyadenosine at 200 μg/ml and 750 μg/ml respectively. The percentage of the ribosomes in polysomes, another measurement of the number of polysomes, was 28% for normal embryos and 19% for embryos in 750 μg 3′-deoxyadenosine/ml. These results suggest that the entry of maternal mRNA into polysomes is not dependent on the poly-A formation which occurs at fertilization.

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Afferent activity in the neural control of the heart of Busycon canaliculatum. HAROLD NEAL AND KIYOAKI KUWASAWA.

Previous work on the heart of *Busycon canaliculatum* has shown that spontaneous activity continues in the cardiac nerve following section between the pseudoganglion and the right visceral ganglion. Furthermore, contractions of the heart induced bursts of activity in the cardiac nerve. This activity has been interpreted as afferent. In the present study two series of experiments have been performed to ascertain whether or not this activity is truly afferent and to determine what control mechanisms may depend on this activity. Initially, branches of the cardiac nerve within the heart were stimulated electrically. Such stimulation provided correlated compound action potentials in both left and right parietal-visceral connectives as well as in the cardiac nerve between the pseudoganglion and visceral ganglion. Activity was also recorded in other branches of the cardiac nerve within the heart. This presumably efferent activity was found in all branches of the nerve independent of which branch was stimulated, indicating homogeneity of all branches. Section of the pseudoganglion to visceral ganglion connective resulted in increased amplitude of some but not all compound action potentials, possibly due to removal of an inhibitory effect arising from the visceral ganglion. Later experiments examined nerve activity initiated by applying a quick stretch to the heart. Activity was not recorded in the left parietal-visceral connective but otherwise could be traced to all points previously determined. Release of the stretch also produced bursts of activity in the pseudoganglion-visceral ganglion connective and in the cardiac nerve within the heart. Clearly, afferent activity is involved in a complex control system with reflex pathways through the pseudoganglion. Possible inhibitory mechanisms arise from the visceral ganglion. The recording of activity distal to the visceral ganglion also suggests that control mechanisms may exist in higher centers.

Some effects of compound D-600 at the squid giant synapse. TERRENCE L. PENCEK.

Compound D-600 was first shown to block excitation-contraction coupling in mammalian cardiac muscle by blocking in inward Ca^{++} current without affecting the inward Na^{++} current, excitability, or the upstroke velocity of the cardiac muscle action potential. Recently two phases of inward Ca^{++} current have been shown by the aequorin techniques in squid giant axon: the early phase is Tetrodotoxin sensitive, the late phase is TTX-insensitive, unaffected also by tetraethylammonium, but blocked by other divalent cations (*i.e.*, Ni^{++}, Co^{++}, Mn^{++}, Mg^{++}) and D-600. D-600 also blocks the inward Na^+ current but does not affect inward or outward K^+ current. To test D-600 effectiveness in excitation-transmitter release coupling, the frog sartorius muscle preparation was used. End plate potentials decreased by 50–70% in 30 minutes with 10^{-4} M D-600. Miniature end plate potential amplitude remained constant while frequency decreased slightly. The reduction in EPP was probably therefore not due to some postsynaptic effect.

The squid giant synapse requires a greater D-600 concentration because of the increased Ca^{++} concentration in ASW. Solvent problems were first encountered: greater than 5 mM dimethyl sulfoxide or greater than 10 mM ethanol reduced the action potential and could not be used. When D-600 was applied at 5×10^{-4} M the presynaptic spike decreased in amplitude before postsynaptic alteration occurred. In ASW containing 2×10^{-7} M TTX, the input-output relationship (presynaptic peak depolarization *vs.* log postsynaptic potential) was shifted to the right, an indication of less efficient synaptic transfer function. This same shift is seen when reducing the extracellular Ca^{++} concentration. In a TEA injected preparation, 10^{-3} M D-600 blocked the "on" and "off" PSP. We suggest that there is a parallel between the late Ca^{++} channel and excitation-transmitter release mechanism and that a selective Ca^{++} antagonist will help to define the possible Ca^{++} ionophore involved.

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Calcium binding sites in synaptic vesicles of the frog neuromuscular junction. A. L. Politoff, S. Rose and G. D. Pappas.

The electronmicroscopic visualization of insoluble materials that bind electron dense ions can be accomplished by fixation in the presence of such ions. Oschman and Wall (1972, *J. Cell Biol.,* **55**: 58) have shown that fixation in the presence of Ca^{++} results in the appearance of electron dense precipitates on some cell membranes. Electron probe analysis shows that they contain high concentrations of Ca^{++} (Oschman and Wall; Hillman and Llinas, personal communications). Precipitates were observed when neuromuscular junctions were fixed in the presence of 5 or 90 mM of $CaCl_2$. In thin sections, between 50 and 80% of synaptic vesicle profiles contained a dense particle of 50–70 Å diameter, suggesting that there is one particle per vesicle. About 42% of all particles seemed to be touching the vesicle membrane. A mathematical model based on the assumption that the particle is touching the membrane predicts that the probability of seeing a particle touching the membrane, given vesicles of 500 Å diameter and an image resolution of about 20 Å, should be near 40%. If the particles were distributed randomly inside the vesicle, the same probably would be less than 1%. It is concluded that the vesicles contain Ca binding materials that are attached to or form part of the membrane, at least after fixation. If the particles exist *in vivo,* the vesicles are polarized and anisotropic. Ca deposits were observed also in mitochondria, sarcoplasmic reticulum and postsynaptic membrane.

It may be postulated that the Ca that enters the presynaptic process before the release of transmitter binds to a specific region of the synaptic vesicle membrane as a preliminary step to transmitter release.

A chlorpromazine-sensitive, high affinity uptake system for L-noradrenaline in synaptosomes from the optic lobe of squid (Loligo pealei). H. B. Pollard, V. A. Bohr, M. J. Dowdall and V. P. Whittaker.

Transmission at adrenergic synapses is terminated by the reuptake of transmitter into the presynaptic terminal. This process can be studied in synaptosomal preparations. Synaptosomes from squid optic lobes have proved useful for the study of choline uptake; an uptake system for L-noradrenaline has now been found in the same preparation. Squid synaptosomes, at 4° and 25° C, were found to take up ^{3}H-L-noradrenaline in linear fashion for up to one hour. The system has a K_m of 0.4 μM and a V_{MAX} at 25° C of 40 picomol/mg protein per minute. Uptake was studied between 4° and 25° C, and Arrhenius plots of the velocity were linear over this range. The activation energy was found to be 10.2 kcal/mole. The system was found to be stereospecific for the L-isomer of noradrenaline. Structural analogues such as serotonin, adrenaline, octopamine and tyrosine did not compete with L-noradrenaline for uptake by the system. By contrast, uptake was markedly inhibited by DNP, KCN and ouabain in 10^{-4} M concentrations, suggesting a metabolic energy requirement. In addition, uptake was found to be competitively inhibited by low concentrations of chlorpromazine with a K_i of 2.5×10^{-8} M. These results suggest that L-noradrenaline is a transmitter in the squid central nervous system. Furthermore, the uptake system for L-noradrenaline has certain similarities to that found in mammalian preparations. For example, the K_m is similar to that for rat brain hypothalamus, while the V_{MAX} of the squid is somewhat higher. Chlorpromazine inhibition is also found in rats. We conclude that the squid synaptosome preparations are a useful system for the study of adrenergic mechanisms in the central nervous system and because of its much higher rate of uptake may present certain advantages over mammalian synaptosomes in such studies.

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Predicting gene frequencies in natural populations. II. *The genetic and physiological basis of protein polymorphism.* DENNIS A. POWERS.

The high proportion of protein polymorphisms in natural populations has created heated literary debate concerning the theoretical foundations of molecular evolution. The major point of contention is whether the majority of molecular evolution has taken place by random genetic drift or natural selection. Since present estimates of mutation rates, genetic load, effective population size and evolutionary rates are inexact, we have addressed our research efforts toward studying structural and functional properties of allelic products in relation to environmental variables. Our approach has been to examine the enzyme kinetics of various phenotypes for a number of polymorphic loci in *Fundulus heteroclitus* as a function of temperature. On the basis of the relative behavior of these allozymes, as a function of temperature, we predict trends in gene frequencies for natural populations of killifish living under different thermal environments (*e.g.*, different latitudes). Afterward, we test our prediction by sampling natural populations along the east coast of the United States. So far all of our predicted trends have been confirmed. In addition, appropriate genetic crosses have been done for all the phenotypes to establish the genetic inheritance of the phenotypes.

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Heat production of Arbacia *eggs revisited.* E. J. PROSEN AND K. S. COLE.

Heat production is an important aspect of the thermodynamics and kinetics of the initiation and subsequent metabolic—and perhaps physical—processes of growth. Tremendous advances in speed, sensitivity, and accuracy of heat measurements have come about from the use of modern techniques and semiconductor materials. Yet little, if anything, has been done on such simple systems as marine eggs since the 1924 measurements of Rogers and Cole.

We have repeated that work with the advanced microcalorimeter of the National Bureau of Standards (Prosen, 1973) which has been developed and used in clinical studies of enzyme reactions and bacterial growth. The instrument had been modified to accommodate a larger 3.4 ml reaction vessel to adapt it for biological work. This isothermal, heat-flow instrument measures the heat production of two solutions (0.75 ml and 1.50 ml) before and after mixing with an output signal of 0.2 volt/watt or 90 μcal/hr (corresponding to 0.1 μk), and with a response half-time of 2 minutes.

Since a constant-temperature room was not available it was necessary to place additional insulation around the calorimeter to limit baseline drift to 3 μW/hr or less. The original plan was to measure the heat production of several thousand *Arbacia* eggs and corresponding sperm in the separate compartments of the reaction vessel and to follow it—after fertilization by mixing—for a few hours or several cell divisions. This has been accomplished but not as yet satisfactorily since no greater than 90 per cent fertilization has been achieved in the vessel. It was first found that no fertilization or development took place in the plastic reaction vessels used, although controls in glass gave over 95 per cent. A glass vessel with ports sealed with paraffin also proved unsatisfactory. Finally the original plastic vessels operated properly (90% fertilization) after flushing them with sea water for several days. The vessel ports are closed with o-ring seals.

Egg and sperm counts are incomplete, but our preliminary results are as follows: (all measurements were made in natural sea water containing 0.0001 molar EDTA and pH 7.8). Unfertilized eggs gave about 0.3 μW/1000 eggs after 1 hour with a gradual drop with time. Prefertilized eggs gave about 0.6 μW/1000 eggs after 1 hour and with some small variations and a gradual drop with time. Sperm heat was also measured but results are not complete. Fertilization runs in the calorimeter gave similar heats for the unfertilized and the fertilized separate runs above and showed no burst of heat upon fertilization. Fluctuations suggest a possible correlation with stages of development.

There is no immediate explanation for the fact that these values are about twice as large as those of Rogers and Cole. However, different conditions were used and in one experiment with greater loading (50,000 eggs/ml in place of the usual 10,000 eggs/ml) we obtained their value. They used 100,000 eggs/ml. We conclude that it should not be difficult to extend the range of experiments to include measurements of fertilization and early development of a variety of marine eggs.

Calcium associated with the plasma membrane in squid axon. ROBERT PRUSCH, BETTY J. WALL AND JAMES L. OSCHMAN.

Previous electron microscope studies have demonstrated that fixation of squid axons in a mixture of glutaraldehyde and calcium results in formation of opaque deposits about 500 Angstroms in diameter on the inner surface of the axolemma. Microprobe analysis reveals that the deposits contain calcium and phosphorous. The present study is the first in a series of investigations on the possible physiological significance of the apparent binding of calcium to the axolemma. On the basis of the size of the deposits and their frequency (average 2.5/micron) it can be estimated that the total amount of calcium associated with the membrane is on the order of 0.1 mM/kg wet weight of axoplasm. The calcium content of extruded axoplasm is about 1 mM/kg wet weight (flame photometry). This value for *Loligo pealei* is somewhat higher than that obtained by Baker and colleagues for *Loligo forbesi.* It is generally thought that only a small fraction of axoplasmic calcium is ionized (Baker and colleagues estimate 0.0003 mM/kg for *Loligo forbesi*). The membrane thus appears to be able to bind much more calcium than is present in ionized form, indicating that membrane sequestration could be significant in buffering intracellular calcium concentration.

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Restoration of the birefringence of the mitotic apparatus of Spisula *eggs* in vitro *by the addition of chick brain tubulin.* LIONEL REBHUN, PAUL LEFEBVRE AND JOEL ROSENBAUM.

Recently, methods have become available for the assembly of microtubules *in vitro* (1972, Weisenberg, *Science,* 177: 1104). By use of these methods together with new procedures for the isolation of the mitotic apparatus we were able to restore and augment the birefringence of isolated *Spisula* mitotic apparatus (MA) which had been depleted of microtubules by cold treatment. This was done by adding purified chick brain tubulin subunits to the tubule-depleted MAs. Chick brain tubulin was purified by repetitive polymerizations at 37° and depolymerizations at 4° C. The MAs were isolated from KCl-activated *Spisula* eggs in a modified microtubule polymerization medium (PM) containing low concentrations of Triton X-100 and the proteolysis inhibitor TAME (tosyl-arginine methyl ester). The MAs isolated by this procedure were stable at room temperature and could be depleted of their microtubules by cold treatment or by the addition of calcium. The isolated MAs were washed once in PM and then suspended in PM with chick brain tubulin (9 mg/ml). Control MAs were suspended in PM without tubulin or in tubulin plus 10^{-3} M colchicine. The birefringence of the experimental MAs (PM + tubulin) increased by at least 40% within 15 min at 25° C. Control and experimental MAs were then placed in the cold to dissociate the microtubules. The cold treatment was always begun within 10 min of MA isolation as the cold lability of the mitotic tubules decreased rapidly with time. Within 30 min, most of the birefringence of the MAs had disappeared; the temperature was then raised to 30° C. Experimental MAs (PM + tubulin) recovered 90% of their normal birefringence within 15 min. Over the next hours many of the MAs increased in length up to two times compared to normal MAs. The control MAs in PM alone and in PM + subunits + colchicine did not recover their birefringence. The experiments show that heterologous microtubules can be reconstructed *in vitro* within a MA remnant depleted of its microtubules. Similar experiments are in progress utilizing *Spisula* (homologous) tubulin. Other work is being directed toward determining if microtubule assembly on the isolated MAs is localized or if it occurs throughout the MA.

Part of this study was performed in Fertilization and Gamete Physiology Training Program at the Marine Biological Laboratory (NIH grant 5-T01-HD00026-12).

The ontogeny of the localizations which specify the apical tuft and the gut in Cerebratulus lacteus. RICHARD REESE AND GARY FREEMAN.

The region of the *Cerebratulus* egg which forms the apical tuft is derived from the pole of the egg where the polar bodies are formed (the animal pole) while the part of the egg which forms the gut is derived from the opposite pole of the egg (the vegetal pole). During normal development the differential division which separates these two regions occurs during the third cleavage which is the first equatorial division.

N. Yatsu has argued that the localizations of developmental potential which are segregated during the third cleavage are set up gradually over the 2 hour period between fertilization and the first cleavage. Yatsu's argument was based on experiments in which equatorial cuts were used to make nucleated egg fragments at different stages between fertilization and the first cleavage. We have repeated Yatsu's experiments this summer.

The apical tuft can be easily identified cytologically. The gut was identified on the basis of its tissue specific esterase activity. This enzyme was monitored histochemically by using 5-bromoindoxyl acetate as a substrate. This enzyme activity first appears at 24 hours of development (18° C); the pH optimum is 8.

Eighty to one hundred per cent of the animal halves of the *Cerebratulus* egg which are isolated at different times prior to the first cleavage produce guts. About 15 minutes prior to the first cleavage there is a rapid decline in the percentage of cases that will form a gut; by the 2-cell stage the animal half has lost its ability to form a gut. Ninety to one hundred per cent of the vegetal halves of the *Cerebratulus* egg which are isolated at different times up to the 4-cell stage produce apical tufts. At the 8-cell stage the vegetal half loses its ability to support apical tuft formation.

This work is supported by N.I.H. grant GM-20024-01.

Is mesosin another universal fibrous protein? ROBERT V. RICE AND W. J. ADELMAN, JR.

Mesosin is the name suggested for the protein or proteins from which three relatively high molecular weight polypeptide chains are derived by SDS acrylamide gel electrophoresis. In 5.7% gels three bands of 150,000, 135,000, and 100,000 daltons are observed from the electrophoresis of purified intermediate (10.0 nm) filaments of mammalian smooth muscle. All smooth muscles so far examined contain these intermediate filaments which are closely associated with dense bodies. The intermediate filaments are hollow and the amino acid compositions of the purified filaments (obtained from sliced gels of each band) do not correspond with any known fibrous protein. Other types of cells also show intermediate filaments which are morphologically similar. These include axons, BHK21, and myotubes. SDS gel electrophoresis of extruded axoplasm from *Loligo pealei,* giant axons show 12 bands. The 130,000 dalton band has small satellites on each side at approximately 150,000 daltons and 100,000 daltons. The highest concentration is at 130,000 daltons just as in smooth muscle. Until amino acid analyses and peptide mapping are done on both smooth muscle mesosin and the axoplasm gel bands no conclusion can be drawn as to the universality of mesosin.

Two-step arrest of sea urchin morphogenesis by tosyl lysine chloromethyl ketone. PAUL ROSENBERG AND ROBIN WALLACE.

Tosyl lysine chloromethyl ketone (TLCK) is a synthetic, irreversible inhibitor of trypsin-like proteases. *Arbacia punctulata* embryos were treated for 2 hr at various times with TLCK at a concentration of 0.27 mM in MBL formula sea water plus 25 mM Tris at pH 7.0. Embryos pulsed in the first five hours post fertilization did not develop further. Embryos pulsed prior to hatching (5–7 hr p.f.) went on to hatch into swimming blastulae but did not gastrulate.

Embryos pulsed between hatching and the completion of gastrulation (16 hr p.f.) gastrulated normally, but arrested at the onset of the prism stage. This latter effect was reversible, and embryos so treated developed into prisms 10–20 hr behind controls, depending on the time of the pulse: the later the pulse, the longer the lag. Embryos treated after the onset of the prism stage (19 hr) developed no further for at least 40 hr.

In order to pursue the question of whether the effect of TLCK is due to a specific action on a protease, embryos were exposed to TLCK in the presence of the reversible protease inhibitor benzamidine, which blocks inactivation of trypsin by TLCK *in vitro*. Using a 3 hr pulse of TLCK and benzamidine in concentrations up to 2×10^{-2} M, no difference was observed between the experimental group and TLCK controls. Embryos were radialized by exposure from hatching to 5×10^{-3} M benzamidine alone, but were essentially unaffected by a 3 hr pulse during gastrulation. Soybean trypsin inhibitor at concentrations up to 1 mg/ml had no effect on development.

In *E. coli*, glutathione has been shown to antagonize the effects of TLCK due not to TLCK itself but to a ketoaldehyde breakdown product which would appear to be a good, non-specific SH alkylating agent (T. Rossman, C. Norris, and W. Troll, in press, *J. Biol. Chem.*). In our studies, 5×10^{-4} M reduced glutathione completely antagonized the effects of TLCK when administered simultaneously, while glutathione supplied continuously following the TLCK pulse had no effect. L-cysteine and dithiothreitol also antagonized the effect of TLCK when administered simultaneously. Oxidized glutathione had no effect at any time.

It therefore seems that the TLCK effect is mediated by the action of a breakdown product on SH groups in the embryo, since it is antagonized by several SH reagents. This does not exclude the possibility that an SH-dependent protease is involved.

This study was performed in the Fertilization and Gamete Physiology Research Training Program at the Marine Biological Laboratory (NIH 5-T01-HD00026–12).

Life-habits of Cumingia tellinoides: *an example of convergent adaptation.* W. D. RUSSELL-HUNTER AND JAY SHIRO TASHIRO.

The bivalve order or superfamily Tellinacea encompasses four families, made up of deposit-feeding genera with separate long extensible siphons, a pallial cruciform muscle, a blade-like foot for rapid burrowing, and rather large labial palps. Two of the families within the group: the TELLINIDAE, to which the genera *Tellina* and *Macoma* belong, and the SEMELIDAE, to which *Semele*, *Abra* and *Cumingia* belong, can be separated using phyletically conservative features. The tellinids have an elongate external ligament with rather weak hinge-teeth, while the semelids have an internal functional ligament on a chondrophore and strong cardinal and lateral hinge-teeth.

Within the tellinids, several species of the genus *Macoma* have long been known to live on their sides in sediments, and show characteristic posterior twists to the shell valves and asymmetric pallial sinuses.

The local semelid, *Cumingia tellinoides*, lives in muds associated with eel-grass. Using artificial deposits of the pure mineral cryolite (which has a refractive index close to that of sea water, see Josephson and Flessa, 1971, *Biol. Bull.* **141**: 392; 1972, *Limnol. Oceanogr.*, **17**: 134), it proved possible to photograph specimens of *Cumingia* both in the initial stages of burrowing, and also buried in the sediment extending their siphons in their normal feeding movements. Behavior varies in *Cumingia*, but burrowing experiments using natural sediments resulted in 14% vertical, 10% at about 45° on their sides and 76% nearly horizontal on their sides. Obvious asymmetry of the pallial sinus occurs in at least 49% of shell-valve pairs, and a clear directional twist of the posterior end in about 23%, some deformation being obvious in more than 73%.

Obviously this habit of lying on one side in the sediment, as done by both *Macoma* and *Cumingia*, is associated with a much less active way of life than that of *Tellina* on the one hand or of *Semele* on the other. The habit, and its associated structural asymmetries, must have been evolved independently in the tellinid, *Macoma*, and in the semelid, *Cumingia*. It is therefore another example from bivalve lineages of apparently convergent adaptation, comparable to the polyphylogeny of the limpet-form in gastropods or of tracheae in arthropods.

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Premature cleavage following prefertilization exposure of Arbacia *eggs to NH_4OH.* Ronald C. Rustad and Rosalind Goldman.

Exposure of unfertilized *Lytechinus* eggs to NH_4OH in sea water is known to gradually change the membrane potential from −10 mV to −60 mV. This treatment leads to DNA synthesis, chromosome condensation and nuclear membrane breakdown.

In the present experiments *Arbacia* eggs were exposed to NH_4OH and washed either before or after fertilization. Brief treatments sometimes delayed cell division, optimal ones induced premature cleavage and long exposures often delayed or blocked mitosis. The treatments generally led to a reduction in the amount of mitotic delay induced by γ-irradiating the sperm, eggs or zygotes. The percentage of reduction in radiation-induced mitotic delay was consistently greater when the sperm rather than the egg or zygote was irradiated. In fact, eggs treated with NH_4OH and fertilized with irradiated sperm frequently divided before control cells.

In vivo observations on unfertilized *Arbacia* eggs indicated that NH_4OH induced the formation of astral material and the disappearance of the nucleus. There was usually no elevation of a fertilization membrane or formation of a hyaline layer. Nonetheless, two types of pseudofurrows were found. Some eggs exhibited a radially symmetrical cleavage, while other eggs elongated into a crescent shaped figure in which a unilateral furrow cleaved the cytoplasm. Cleavage could be blocked by cytochalasin B, an observation suggesting that 50 Å microfilaments may be involved in these types of cleavage as well as in normal cytokinesis. After the nuclei reappeared they swelled and developed large refractile nucleosomes.

These observations indicate that prefertilization exposure of sea urchin eggs to NH_4OH can shorten the postfertilization mitotic cycle. In addition, treatment with NH_4OH can cause unfertilized eggs to exhibit unusual forms of karyokinesis and cytokinesis.

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A study of the innervation of the radular protractor muscle of Busycon canaliculatum. J. W. Sanger and R. B. Hill.

Each radular protractor muscle is innervated by a nerve arising from the cerebro-buccal connective. The nerve trunk enters the proximal end of the muscle and runs parallel to the long axis of the muscle and to the central artery. A layer of connective tissue and epineural muscle cells surrounds the nerve trunk. Subdivisions of the main nerve trunk branch laterally into the radular protractor muscle losing their epineural muscle coat. Further subdivisions of the branches produce single axons which can be observed among the muscle cells, but no specialized motor endings were observed. Within the nerve endings are two types of synaptic vesicles: agranular (clear) and granular (dense). The granular vesicles are larger, ranging in diameter from 800 to 1350 Å (mean 970 Å). The clear vesicles vary in diameter from 500 to 1000 Å (mean 630 Å). The ratio of agranular to granular vesicles within a single nerve ending varies widely. The mixing of different types of synaptic vesicles within a nerve ending is similar to what has been reported in the anterior byssus retractor muscle of *Mytilus edulis.* If the different types of vesicles contain different neurotransmitter chemicals then perhaps a single nerve ending releases more than one neurotransmitter. This is suggested by previous observations on the radula protractor muscle indicating that both low concentrations of acetylcholine and low concentrations of serotonin can potentiate the excitatory effects of nervous stimulation.

Protein polymorphism of the seastars Asterias forbesi *and* Asterias vulgaris: *an evolutionary paradigm for the Cape Cod marine microcosm.* Thomas J. M. Schopf and Lynda S. Murphy.

The warm-water, southern Cape Cod seastar *Asterias forbesi* and its "twin" the cold-water, northern Cape Cod *A. vulgaris* are normally characterized by 7 prominent phenotypic differences. However hybrids occur in localities where the range of the two species overlap, such as bays north of Cape Cod, and offshore regions south of Cape Cod. Analysis of 9 enzyme and protein systems revealed 27 loci in *A. forbesi* and 26 loci in *A. vulgaris,* with 19–72 individuals used to characterize each band system. Using the method of Nei (1972, *Amer. Natur.,* **106**: 283), 67% of the gene loci are held in common by both species.

How did this situation originate? We propose that *A. forbesi* and *A. vulgaris* are derived from a late Tertiary wide-spread species that was subjected to geographic partitioning during the Pleistocene. At least 70 m of the present relief on Georges Bank is due to glacial deposits, and, in addition, pre-glacial sea level was about 60 m higher than present sea level. With formation of glaciers causing lowering of sea level, and the coincident emergence of a land barrier (Cape Cod-Georges Bank), the geographic range of a widespread species would have been divided. We contend that northern local populations evolved into the cold-water *A. vulgaris* whereas southern populations led to the present, shallow-water temperate species *A. forbesi.* According to this evolutionary model, the present distribution, ecology, genetic similarity and hybrid formation of these seastars is understandable. This also provides a marine counterpart to the similar cases of terrestrial speciation. Finally, review of literature on other marine species has revealed pairs of species with contrasting ecology in the amphipods, crabs, clams, hydroids, mysids, ostracods and ectoprocts. To a significant extent, Cape Cod may have "caused" its own species diversity.

Function of cortical granule protease: re-fertilization of Arbacia *eggs fertilized in soybean trypsin inhibitor.* H. SCHUEL, F. J. LONGO AND W. L. WILSON.

Cortical granules of unfertilized sea urchin eggs contain a tryptic protease (Schuel *et al.*, 1973, *Develop. Biol.,* **34**: in press). This enzyme is involved in the establishment of the block to polyspermy at fertilization. *Arbacia* eggs fertilized in the presence of soybean trypsin inhibitor (SBTI) become heavily polyspermic. SBTI retards the following processes in treated eggs at fertilization: discharge of the cortical granules, dispersal of the secretory product released from the cortical granules into the perivitelline space, and separation of the vitelline membrane from the egg surface to form the fertilization membrane. Numerous small loci at the egg surface (cortical projections), from which the fertilization membrane fails to elevate, remain until about 10 to 15 min after the initiation of the cortical reaction. Sperm continue to penetrate treated eggs through the cortical projections until the elevation of the fertilization membrane has been completed. Eggs re-inseminated in SBTI show a progressive decrease in polyspermy during this period. Thus the cortical projections are the sites at the egg surface for sperm penetration during re-fertilization in the presence of SBTI.

The sea urchin egg, the classical system for studying embryonic development, is also an ideal model system for studying secretion per se. On the basis of our work on the cortical granule protease, we propose that tryptic proteases function in the discharge of secretory granules in all cells.

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The sulfur requirement for the completion of the life cycle of Chlorella pyrenoidosa *chick (Emerson strain).* STUART G. SIDDELL, ROBERTO GONZALES, ROBERT C. HODSON AND JEROME A. SCHIFF.

Autospores of *Chlorella* deprived of a sulfur source fail to complete enlargement and do not divide. A utilizable sulfur source supplied to sulfur-starved cells induces synchronous enlargement and division after 20–30 hours. Sulfur-starved cells of wild-type, which are capable of sulfate reduction, and mutants Sat_1^- and Sat_2^- which are blocked for sulfate reduction, require a minimum of 10^{-5} M sulfur to undergo cell division. The increase in cell number due to daughters is dependent on the concentration of sulfur added. Thiosulfate, sulfate or methionine will serve as a utilizable sulfur source for wild-type cells. The mutants will not complete the life cycle on sulfate but will do so on thiosulfate or methionine. This indicates that the limitation imposed by sulfur starvation is not solely in the formation of sulfate esters but must also involve a block in protein synthesis. Consistent with this, cycloheximide (50 μg/ml) inhibits completion of the life cycle in the presence of a completely utilizable sulfur source.

Incorporation studies employing $^{35}SO_4^{2-}$ are also consistent with this interpretation. Wild-type cells take up almost all the supplied radiosulfate, which appears in the soluble pools, the lipids, the polysaccharides and the proteins. Sat_2^- incorporates comparable amounts of label into all fractions except protein. Thus Sat_2^- is synthesizing sulfolipid and polysaccharide, like wild-type, but is not making protein. In Sat$_1^-$, which takes up less label than the other two types of cells, label appears only in the soluble pools; negligible incorporation into the lipid, polysaccharide and protein fractions is observed. Protein synthesis seems therefore, to be the major limitation on completion of the life cycle during sulfur starvation.

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Light-evoked responses from the ventral parolfactory vesicles of Loligo pealei. LINDA SPERLING, J. E. LISMAN AND ANTHONY GODFREY.

Light micrographs of this preparation show ellipsoidal (*ca.* 30 micron) cells packed into 200 micron clusters. The cells can be impaled with 30 megohm micropipettes, give resting potentials of about −40 mV, and can be maintained for 5 hours in oxygenated seawater (10 mM dextrose, 10°–15° C). When two cells of the same cluster were impaled simultaneously, partial electrical coupling was recorded in 1 out of 4 pairs. Cells dark-adapted to absolute threshold within an hour after exposure to bright stimuli. Receptor potentials were depolarizing, with a maximum amplitude of 50 mV. Depolarization of the cell by current injection decreased the amplitude of the receptor potential; hyperpolarization increased it. The response to 1 sec stimuli intense enough to isomerize at least 1% of the rhodopsin were followed by an after-depolarization which could last as long as 7 minutes. Flashes delivered during the after-depolarization evoked biphasic responses consisting of an initial depolarization (100 msec) followed by a hyperpolarization (100 msec). The peak voltage of the hyperpolarizing component of this response could be more negative than resting potential. A bridge circuit was used to inject current and thereby to measure membrane resistance in the dark (20 megohms) and the pseudo-time constant (10 msec). Even a few mV depolarization resulted in repetitive firing of 2 msec spikes, 1 to 2 mV in amplitude. Dim illumination (4×10^7 photons/sec/cm^2 at 530 nm) evoked, on the average, one quantum bump (2 mV, 100 msec) per second. The bump frequency increased linearly with light intensity, and bumps could be transiently abolished by a bright adapting stimulus. Assuming a driving force of 50 mV, the peak conductance-change during a quantum bump is 2000 pmho.

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Gasterostome trematodes of the Woods Hole region. HORACE W. STUNKARD.

Linton [1898, *Proc. U. S. Nat. Mus.*, **20**: 507; 1900, *Bull. U. S. Fish. Comm.*, **19**(1899): 267; 1901, *Bull. U. S. Fish. Comm.*, **19**(1899): 405] described adult gasterostomes from various marine fishes taken in Woods Hole, Massachusetts area, and (1905, *Bull. Bur. Fish.*, **24**: 323) reported and figured encysted juvenile specimens from *Menidia menidia* at Beaufort, North Carolina. He (1940, *Proc. U. S. Nat. Mus.*, **88**: 1) summarized his findings, allocated the worms to the genera *Gasterostomum, Prosorhynchus,* and *Nannoenterum,* but the determinations and dispositions have been questioned repeatedly. In the description of *Prosorhynchus gracilescens* (Rudolphi, 1819), he included adult specimens from gars, *Strongylura marina*, and encysted juveniles from *M. menidia,* that according to Hopkins (1954, *Parasitol.*, **44**: 333) should be assigned to *Rhipidocotyle transversale* Chandler, 1935 and to *Rhipidocotyle lintoni* Hopkins, 1954. Hopkins found adults of both *R. transversale* and *R. lintoni* in *Strongylura marina* collected at Gloucester Point, Virginia, Grand Isle, Louisiana, and Port Aransas, Texas. Encysted juveniles of *R. transversale* were found in *Menidia* spp. from Louisiana and Texas.

Encysted juveniles of both *R. transversale* and *R. lintoni* are common in *M. menidia* at Woods Hole. The incidence of infection varies from 10 to 40 per cent in different collections. Both species may occur in the same individual fish and in general the two species appear to be present in about equal numbers. They are about the same size. The intensity of infection varies from one to 38 worms in a fish, usually only two or three. They are encysted in muscles, under the skin at the bases of the fins, and in the midventral region posterior to the anus. The cysts are oval to elongate, 0.50 to 1.20 mm in length, about twice as long as broad, and the worm occupies about three-fourths of the cavity of the cyst. In the cysts the worms are cylindrical, tapering toward the ends, but broader anteriorly. Released from the cysts, they are flattened by pressure of a cover-glass and appear larger. They grow to full size in the cysts and differ from adults only in the absence of eggs.

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Difference in fluorescence behavior between positional isomers of aminonaphthalene derivatives applied internally to squid giant axons. I. Tasaki, K. Sisco and A. Warashina.

Various positional isomers of aminonaphthalene derivatives were found to behave very differently in the membrane of squid giant axons. The first example investigated is 8-*amino*-1-naphthalene sulfonate (1,8 AmNS) and 2,6 AmNS. The 1,8 AmNS applied to axons by intracellular injection gave rise to transient changes in fluorescence (*i.e.*, fluorescence signal) during action potentials, while 2,6 AmNS administered internally produced no fluorescence signals. This difference is interpreted in terms of the difference in sensitivity of the two isomers to *solvent polarity*. The fluorescence intensity of 1,8 AmNS is strongly affected by the dielectric constant of the solvent, while that of 2,6 AmNS is not.

8-*Anilino*-1-naphthalene sulfonate (1,8 ANS) has been used extensively as a fluorescence probe. Its isomer, 2,6 ANS, was also found to be a useful probe for the axonal membrane. The fluorescence signals obtained with 1,8 ANS represented a decrease in intensity of poorly polarized emission and had a broad spectrum extending from 400 nm to well beyond 550 nm. The signals derived from 2,6 ANS in squid axons were found to be highly polarized, indicating that both the absorption and emission oscillators are almost perfectly oriented along the long axis of the axon. The spectrum of 2,6 ANS signal was sharp and narrow, terminating abruptly at about 485 nm. This difference in behavior between 1,8 ANS and 2,6 ANS is attributed to the following two physical factors: (1) The lowest-energy absorption band of 1,8 ANS represents two different types of electronic transitions while the absorption band of 2,6 ANS at 365 nm involves only one type of electronic transition, and (2) the transition moment of 2,6 ANS is oriented along the long axis of the molecule while 1,8 ANS does not have a well-defined long axis. The importance of a *longitudinally oriented* macromolecular structure at or near the axon membrane which brings about nearly perfect alignment of 2,6 ANS molecules is emphasized.

Activation of ribosomal RNA accumulation in sea urchin plutei by insulin. Andrea J. Tenner and Tom Humphreys.

Sea urchin embryos advance to pluteus stage without net increase in mass. When the gut is developed and an exogeneous food source is utilized, growth of the pluteus is initiated. Recent studies have shown that growth of cells in culture is stimulated by insulin. One proposed model suggests that insulin interacts with acceptors for an insulin-like protein which stimulates multiplication (Dulak and Temin, 1972, *J. Cell. Physiol.*, **81**: 153). An early event in the initiation of growth of cells in culture is the activation of ribosomal RNA (rRNA) accumulation (C. Emerson, 1971, *Nature New Biology*, **232**: 101). Therefore, measurement of newly synthesized rRNA was used to monitor the growth stimulating effects of feeding and of insulin on sea urchin plutei.

Embryos of *Arbacia punctulata* and *Lytechinus pictus* were allowed to develop to the pluteus stage at a concentration of 100 embryos per ml. Embryos were then concentrated to 1000 embryos/ml MBL sea water and given algae, *Dunaliella tertiolecta* (1000 algae/embryo) or insulin (100 μg/ml). After several hours of incubation the cultures were labeled with ^{3}H-adenosine. *Dunaliella* was always removed from the medium prior to the addition of the isotope. RNA was extracted with phenol-sodium dodecyl sulfate (SDS), layered on 15–30% sucrose density gradient buffered with Tris-SDS buffer, centrifuged at 200,000*g* for 7 hours at 25° C, and the optical density and radioactivity of the resulting fractions were measured and recorded. The specific activity of the ATP pool was determined and used in calculating the rate of rRNA formation.

The results show that when *L. pictus* plutei are fed for five hours followed by a four-hour labeling period, the rate of accumulation of newly synthesized rRNA increases to over 5-fold (30.95×10^{-12} g rRNA/embryo/hr) that of unfed embryos (5.95×10^{-12} g rRNA/embryo/hr).

Plutei incubated in the presence of insulin (bovine pancreatic) show a 2.4-fold increase in the rate of rRNA formation (14.96×10^{-12} g rRNA/embryo/hr), whereas embryos incubated in the presence of the same concentration of bovine serum albumin (100 μg/ml) showed no increase in the rate of rRNA formation when compared to untreated, unfed embryos. Mesenchyme blastula, incubated in insulin under identical conditions as plutei, showed no increase in the rate of accumulation of newly synthesized rRNA. *Arbacia punctulata* plutei did not respond to feeding by five hours, but did increase their rate of rRNA formation 4-fold after fifteen hours of feeding. Stimulation by insulin was not detected during similar incubation periods.

These observations suggest that an endogeneous insulin-like growth factor may participate in mediating the initiation of growth when plutei feed.

This study was performed in the Fertilization and Gamete Physiology Research Training Program at the Marine Biological Laboratory (NIH 5-Tol-HD00026-12).

Fluorescamine as a reagent for quantitating precipitates produced by proteolysis. WALTER TROLL AND MILTON LEVY.

Proteolytic enzymes are generally quantitated by measuring the amino groups liberated by hydrolysis of peptide bonds. Sometimes a precipitate forms in the reaction mixture. Examples of precipitate formation are thrombin acting on fibrinogen and chymotrypsin or rennin acting on casein. Trypsin acting on histones (except F-1) produces precipitates promptly. Such precipitates result from the removal of limited hydrophilic regions of the protein leaving a hydrophobic "core." We have been concerned with developing a sensitive method for trypsin (and for trypsin-like proteases) which depends on the isolation of the precipitates produced by trypsin acting on histone, solution of the precipitates and quantitation of their amount by reaction with fluorescamine (F). F reacts with aliphatic amines to yield intensity fluorescent compounds. The half time of the reaction is 0.1–0.5 seconds. Excess reagent hydrolyses with a half time of 10 seconds and no fluorescent products are formed. A commercial mixture of histones in 0.1 cc containing 5–10 mg/cc is incubated with nanogram amounts of trypsin at pH 7.5 and after a time the reaction is stopped by acid. The precipitate is collected by centrifugation, washed and dissolved in 0.1 cc of 6 M urea. An aliquot is brought into reaction with F and the fluorescence measured in a Farrand fluorometer. Precipitate formation is zero order and proportional to enzyme concentration from 10–100 ng. With this method the trypsin like protease liberated on fertilization of sea urchin eggs can be determined. It is accompanied by material capable of inhibiting trypsin action. The method has advantages over other methods in sensitivity and specificity, *i.e.*, chymotrypsin produces no precipitate with histones. Soluble amino groups in the sample of enzyme do not interfere because the product measured is a precipitate formed from the specific substrate.

Alkaline phosphatase activity in unfertilized eggs of Lytechinus variegatus *after removal of endogenous inorganic phosphate.* ROBIN A. WALLACE, MARK N. HILL AND RONALD J. PFOHL.

Previous studies have shown that the specific activity of alkaline phosphatase increases concomitant with the decrease in endogenous inorganic phosphate during development from the unfertilized egg to the pluteus stage, the correlation coefficient between the variables being −0.91. Addition of various concentrations of exogenous inorganic phosphate repressed alkaline phosphatase activity in a pattern qualitatively similar to the *in vivo* relationship. Pfohl has suggested that this increase in alkaline phosphatase activity may be due either to the release from inhibition or activation of a pre-existing enzyme which, on the basis of electrophoretic evidence, is different from that which is active in the unfertilized egg (1965, *Exp. Cell Res.*, **39**: 496). DEAE-cellulose column chromatography of alkaline phosphatase from different developmental stages indicated that the enzyme elutes in different fractions, suggesting that it may appear in different forms (isozymes) during development.

Unfertilized eggs had their jelly coats removed in pH 5 sea water and were homogenized by sonication for 20 sec in 0.01 M Tris, pH 7.4. The homogenate was dialyzed from 0–8 hr in 8/32 dialysis tubing against the homogenate buffer at 2° C. Each dialysis fraction was then assayed biochemically for (1) alkaline phosphatase activity, by measuring the rate of hydrolysis of p-nitrophenylphosphate, and (2) endogenous inorganic phosphate. Each fraction was also treated with 0.25–16.0 mg/ml of exogenous inorganic phosphate and assayed for alkaline phosphatase activity.

Decreasing levels of endogenous inorganic phosphate had no effect on alkaline phosphatase activity, the activity of the enzyme remaining constant in each dialysis fraction. The addition of various concentrations of exogenous inorganic phosphate to each dialysis fraction resulted in quantitatively similar curves which showed a proportionality between the amount of exogenous inorganic phosphate added and the repression of alkaline phosphatase activity.

The inducibility of alkaline phosphatase may thus involve a more complex mechanism than its repression. This might be germane to considerations of different systems of alkaline phosphatase activity regulation in the unfertilized egg as compared with later stages, since at least two distinct isozymes of alkaline phosphatase are found in the developing embryo. Investigations are under way to more clearly elucidate the factors involved in the complex series of changes taking place in the activity of this enzyme.

This study was performed in the Fertilization and Gamete Physiology Training Program at the Marine Biological Laboratory (NIH 5-T01-HD00026-12).

Studies of microsporidan disease transmission in winter flounder and smelt. EARL WEIDNER.

Two species of food fish, winter flounder (*Pseudopleuronectes americanus*) and smelt (*Osmerus operlans*) suffer devastating annual epidemics of microsporidosis. The infections, which are localized particularly as large colonies within the intestinal submucosa, mesenteries or peritoneal lining, are believed to have considerable effect on population size, especially in the young-of-the-year flounder and smelt.

In microsporidosis in general, resistant spores are the means by which an infection begins. However, feeding *Glugea stephani* spores to uninfected flounder does not initiate the disease. All spores fed to flounder appeared unaltered after passage through the alimentary tract. To follow this, washed spores, placed in short lengths of millipore tube-chambers (0.45 μ porosity) were fed to 4–6 yr-old flounder. After passage, the spores were recovered and viewed by phase microscopy to determine whether the spores were empty, changed or unaltered. Since *G. stephani* spores remained visably unaltered during and after passage through flounder, we began testing Stunkard and Lux's idea that small crustacea, which are part of the young-of-the-year fish's food chain, may serve to transfer the infection from fish to fish. Our results indicated that gammaridean amphipods caused up to 40% of the passaged *G. stephani* spores to become either altered or empty. On the other hand, there were no observable effects on spores after passage through the alimentary tracts of animals such as isopods (*Ligia* sp., *Cyathura polita*), polychaetes, and decapods (*Homarus americanus, Carcinus maenas*).

Our results further indicate that *G. stephani* (from winter flounder) and *G. hertwigi* (from smelt) grow in gammaridean amphipods. No spores were detected in amphipod tissues 5 days after feeding with *G. stephani* or *G. hertwigi;* however, two weeks after feeding, all the animals examined had vegetative and spore-forming phases of microsporidan development localized in muscle tissue. We believe that these crustacea are likely the normal hosts for these parasites; if this is the case, their abundance ranks them as potentially large parasite reservoirs for food fishes.

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Evidence for the localization of an RNA template for endodermal alkaline phosphatase in an ascidian egg. J. R. WHITTAKER.

Histochemically detectable alkaline phosphatase (EC 3.1.3.1) activity (method of Gomori and von Kossa) develops in tissues of the presumptive digestive system in *Ciona intestinalis* embryos; it first occurs between hours 5 and 6 of development (late gastrula) at 18° C. Puromycin, an inhibitor of protein synthesis, prevents the development of the enzyme if it is administered at 5 hours or earlier, but not if administered at 6 hours.

The behavior of cleavage-arrested embryos strongly suggests that a morphogenetic material for alkaline phosphatase development is being segregated differentially during cleavage into particular blastomeres of the embryos. Embryos were arrested at various cleavage stages in 2 μg/ml cytochalasin B and examined histochemically for alkaline phosphatase at 12–14 hours of development. Half or more of the embryos at each stage usually developed alkaline phosphatase in some of the blastomeres. The maximum number of blastomeres developing enzyme in these cleavage-arrested embryos followed the cell lineage pattern for larval endoderm cells established by Conklin (1905): 1 blastomere at the 1-cell stage, 2 at 2-cell, 2 at 4-cell, 4 at 8-cell, 6 at 16-cell, 6 at 32-cell, and 10 at 64-cell.

Actinomycin D (20–120 μg/ml), which inhibits the development of acetylcholinesterase and tyrosinase enzymes in *Ciona* embryos, did not prevent the development of alkaline phosphatase even when the drug was administered from fertilization onwards. Daunomycin, chromomycin A_3, and cordycepin, which like actinomycin D are inhibitors of RNA synthesis, likewise inhibited the other enzyme synthesis but not that of alkaline phosphatase. Apparently an RNA template for larval alkaline phosphatase synthesis is localized in the unfertilized *Ciona* egg and subsequent to fertilization is segregated during cleavage into the endoderm cells of the embryo, where it is translated for alkaline phosphatase at the appropriate time (hours 5–6).

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Filamentous components of isolated squid axoplasm. G. WITMAN AND J. ROSENBAUM.

Investigations were made on the *in vitro* assembly of the microtubules (MTs) of isolated axoplasm from the giant axon of the squid, *Loligo pealei.* When the isolated axoplasm was suspended at 0° C in two to five volumes of the solution used by Weisenberg for the polymerization of brain MTs (*Science,* **177**: 1104), no MTs were observed. However, when the suspension was warmed to 20° C, MTs rapidly assembled. These observations show that squid neurotubules can be assembled *in vitro* under the same conditions as vertebrate neurotubules. Moreover, the axoplasm by itself contains all of the components required for *in vitro* assembly of MTs.

The neurofilaments (NFs) of the isolated axoplasm were also studied. When negatively stained, the NFs usually appeared as long, straight tubes, 60–80 Å in diameter, having a stain-filled lumen 15–20 Å in diameter and walls 25–30 Å thick. NFs often appeared to flatten out along portions of their lengths; in these regions three or four 30 Å strands were observed. Less frequently the NFs frayed apart into their component strands; in these cases four strands were observed. These observations indicate that the NFs are composed of four 30 Å strands.

The NFs were partially purified by a modification of the method of Huneeus and Davison (*J. Mol. Biol.,* **52**: 415) in which the NFs are depolymerized in 2 M guanidine hydrochloride and then repolymerized by dialysis against 0.1 M KCl. Analysis of preparations of reassembled NFs by SDS acrylamide gel electrophoresis showed an enrichment for two proteins having molecular weights of *ca.* 78,000 and 170,000 daltons.

Heavy meromyosin (HMM) binding studies showed that the NFs do not bind HMM. However, the isolated axoplasm did contain a few short filaments which reacted specifically with HMM to form typical arrowhead complexes. Furthermore, a protein which comigrated with rabbit actin was observed in SDS acrylamide gels of isolated axoplasm. These results suggest that actin is present in the squid axon.

This study was performed in the Fertilization and Gamete Physiology Research Training Program at the Marine Biological Laboratory (NIH grant 50T01-HD00026-12). One of us (G. W.) was supported by NIH Postdoctoral Fellowship HD 52732.

Some abiotic properties of near UV-irradiated tryptophan. S. ZIGMAN AND T. YULO.

The ability of preformed near UV photoproducts of tryptophan (trp) to reduce the incorporation of amino acids into isolated dogfish (*Mustelus canis*) eye lens and fertilized sea urchin (*Arbacia punctulata*) egg proteins was investigated.

Fresh lenses were presoaked for 24 hr in near-UV light exposed (3W/cm^2; 365 nm) or unexposed elasmobranch Ringer's medium containing 5×10^{-3} M trp. ^{14}C-amino acid (AA) mixture was added (1.25 μCi/5 ml), incubation continued 24 hr, and AA incorporation into total protein of epithelium and cortex was estimated. Incorporation of ^{3}H-uridine into RNA and ^{3}H-thymidine into DNA was also measured.

Millipore-filtered sea water containing 2.5×10^{-4} M trp was UV-irradiated or kept dark for 20 hr. 50 μg/ml of each was added to separate samples of freshly fertilized eggs in sea water at 3 min postfertilization. Incorporation of ^{14}C-AA into protein, ^{3}H-uridine into RNA, and ^{3}H-thymidine into DNA was measured up to 60 min postfertilization.

Lenses incubated with UV-trp were stained yellow. In media UV-irradiated with trp, 24 hr incorporation of AA into corticol protein was depressed by about 50% over controls, while 75% inhibition occurred in epithelium. Uridine incorporation into cortical RNA was reduced by 32%, while 71% inhibition occurred in epithelium. Thymidine incorporation into cortical and epithelial DNA was lower by 24% and 81%. Uptake of precursors into acid soluble phase was inhibited less than 20% by this exposure to UV-trp.

UV-trp markedly delayed or prevented mitosis in fertilized *Arbacia* eggs and led to abnormal development. Permeability to AA's was inhibited less than 20% by UV-trp. Incorporation of ^{14}C-AA into egg protein was 58%, 17% and 27% lower at 15, 30 and 60 min postfertilization respectively; of ^{3}H-uridine into RNA, was 26%, 62%, and 60% lower; and of ^{3}H-thymidine into DNA, was 40%, 48% and 64% lower, respectively, at the same times.

Near UV-trp thus inhibits several phases of protein synthesis in slowly (lens) and rapidly (egg) growing cells. This suggests either a defect in the ability of precursors to reach sites of incorporation or in enzymes involved in the process. Since uptake appears not to be involved, effects on specific enzymes are now being investigated.

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HISTOGENESIS BY CELLS FROM EMBRYONIC AND HATCHED CHICKS IN GIANT, PLATE-LIKE AGGREGATES CULTURED ON A POROUS MATRIX

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It was demonstrated first by Moscona and Moscona (1952) that monodispersed cells of amniote embryos can reaggregate and then build histotypic structures. With a more refined technique, Moscona showed that these capacities are efficient in cells of 7–10 day old chick embryos but decline with the age of the embryo. In his experimental system retinal cells from 7-day chick embryos formed aggregates of 0.3–1 mm in diameter but cells from 19-day-old fetal chicks failed to aggregate altogether (Moscona, 1961, 1962). Lung cells from 16–18 day old embryos aggregated, but showed poor or abnormal histogenesis (Grover, 1961). Even when implanted on chorioallantoic membrane, late fetal or postnatal mouse cells showed limited aggregation and in only a few cases a trace of histogenesis (Ansevin and Buchsbaum, 1962). These results suggested that the capacity to reaggregate and to reconstruct the tissue of origin might be limited to less advanced embryonic stages. This assumption gained support from Kuroda's work (1968) in which the aggregation of cells from older embryos was enhanced by medium that had been conditioned by cells of the same organ from younger developmental stages. Previous experiments with cell aggregation in other laboratories resulted in rapid histotypic reconstruction; however, the aggregates were not maintained in culture for more than 4–6 days.

This report reinvestigates the dependence of aggregation and histogenesis on the age of the cells by means of a new technique, the tissue plate culture (Ansevin and Lipps, 1973). The technique promotes formation of gigantic aggregates (tissue plates) from cells of more advanced developmental stages and sustains their organotypic organization for a longer time than usually is feasible with conventional methods of organ culture.

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MATERIALS AND METHODS

Suspensions were prepared from cells of 14- or 18-day-old chick embryos, and, also, from newly hatched, 2-day-old and 4-day-old chicks. Kidneys or lungs from several animals were pooled in Tyrode salt solution, minced with fine knives and transferred to test tubes. After several rinses and a 10-minute incubation in Calcium-and-Magnesium-free Tyrode (CMF) the tissue preparations were washed with a 1–3% solution of trypsin (Nutritional Biochemicals) in CMF and incubated in a fresh change of trypsin solution for 20 minutes at 38 degrees Centigrade. After that, the tissues were transferred to the culture medium and dispersed by in and out pipeting with a capillary pipet. The preparation was left for several minutes for large undispersed tissue fragments to settle by gravity. The supernatant was transferred to another tube and further dispersed by repeated pipeting. This was followed by brief centrifugation at 20 g to remove undispersed tissue fragments. After this treatment it was found that the supernatant suspension consisted predominantly of single cells among which cell clumps of varying sizes and varying degrees of compactness were present. More culture medium was then added to the sedimented cells and the process of dispersion and centrifugation was repeated several times. Finally, all the supernatants were pooled and a sample drop again was examined under the microscope. This preparation was then centrifuged at 300 g for 15 minutes. After removing the supernatant, the compacted cells were resuspended in several drops of fresh culture medium and placed on top of a porous matrix in plastic Falcon petri dishes 50 mm in diameter. A piece of phenolic sponge—available from many florists—("Oasis," Smithers Co., P.O. Box 118, Kent, Ohio) was used as a matrix for the cells because of its hydrophilicity and the facility with which it could be sectioned on the microtome. The sponge was washed, cut into circles roughly 10 × 3 mm, autoclaved, and presoaked in culture medium. The culture medium consisted of 44% NCTC 135, GIBCO, 44% Tyrode solution, 10% fetal calf serum, 2% chick embryo extract (prepared in this laboratory), 50 units of penicillin, and 50 micrograms of streptomycin per milliliter of the final medium. After the cultures had stood undisturbed for 1–2 hours, 1.5 ml culture medium was added. After the next 48 hours the petri dishes were placed on a Bellco tissue culture rocker operating at the speed of 4 tips per minute throughout the period of cultivation. The cultures were incubated at 38° C in an atmosphere of 95% air and 5% CO_2. The culture medium was changed three times a week. The period of cultivation varied from 0 time (control for aggregation and histogenesis) to 4 weeks. It was followed by fixation of the culture-substrate complex in Zenker fluid, embedding in paraffin, and sectioning at 5 microns either parallel or perpendicular to the surface of the substrate. For staining, the Rapid One-Step methods of Mallory-Heidenhein (according to Humason, 1967) were used.

Although our dispersion procedure yielded cell suspensions in which the integrity of the organ-specific structures (nephric or bronchial tubules) was destroyed, one might suspect that the presence of cell clumps in addition to single cells could have aided reconstructive histogenesis. In order to test whether completely monodispersed cells of the same stages are also capable of undergoing histogenesis, suspensions of exclusively single cells and 2-cell clumps were prepared. This was done by filtering trypsinized suspensions through a Swinny filter assembly with two layers of paper: one layer of "Kimwipe" (Kimberly-Clark Co., Neenah, Wisconsin) and one layer of Japanese "Mullberry drawing paper" (obtained through Andrews/Nelson, Whitehead, New York). These brands of paper gave the most satisfactory results in extensive series of tests of various kinds of paper. Immediately following filtration, only single cells and 2-cell "clumps" could be detected, and decrease in cell population was relatively insignificant. The cells from these suspensions were sedimented by centrifugation and otherwise prepared into cultures as described above for less complete cell dispersions.

Two different types of controls were used in this investigation. One type of controls involved cultures fixed immediately after preparation. This was used for determining the condition of dispersed cells in each experiment before aggregation and histogenesis occurred.

In controls of a second type, dispersed cells were cultured on discs of Millipore filter (assumed to be a two-dimensional, porous substrate) floating on top of the culture medium in Falcon dishes. Only embryonic cells from lung and kidney were used in this type of experiment.

Results

In embryonic and post-embryonic tissues, the cells coalesced extensively in the form of plate-like aggregates that frequently reached about 1 cm in diameter. However, the thickness of the plates never seemed to exceed 0.5 mm.

Kidney on the sponge matrix

When single cell and cell clump suspensions were used, there was no appreciable difference between the extent of aggregation of kidney cells of the following ages: 14-, 18-, 21-days. Histogenesis in plates of cells from 18-day-old chick embryos was as good as that from 14-day-old embryos. The epithelial structures formed ranged from cysts to long cylinders randomly arranged (Figs. 2, 3). Histogenesis in kidney cells from hatched chicks was more limited; nevertheless, tubular structures occurred as shown in Figure 4. These structures were not present in cultures fixed immediately after preparation. The cultures persisted in unchanged condition for 2–3 weeks; afterwards, there was a gradual increase in the amount of connective tissue (Fig. 5), followed by a slow disorganization of epithelial structures. Nevertheless, some tubules persisted in the cell plates for a month or longer. Fully monodispersed cells from 14- and 18-day embryos and from newly hatched chicks aggregated as large tissue plates in which kidney tubules were abundant. For reasons not understood at present, mortality among cells from 2- to 4-day-old chicks was very high. This factor and the existence of an exceedingly high contamination of post-embryonic cells with erythrocytes were very likely responsible for a decline in the formation of large tissue plates by kidney cells of these post-embryonic stages. Small aggregates still formed. Some of them were built of completely solid tissues and were of sufficient size that a tendency to restore histological organization could still be revealed in them by the presence of occasional cystic or tubular structures which showed more or less distinct morphology. Because of this result, we felt that the technique, rather than intrinsic cellular limitations influenced aggregation and histogenesis in postembryonic cells in an

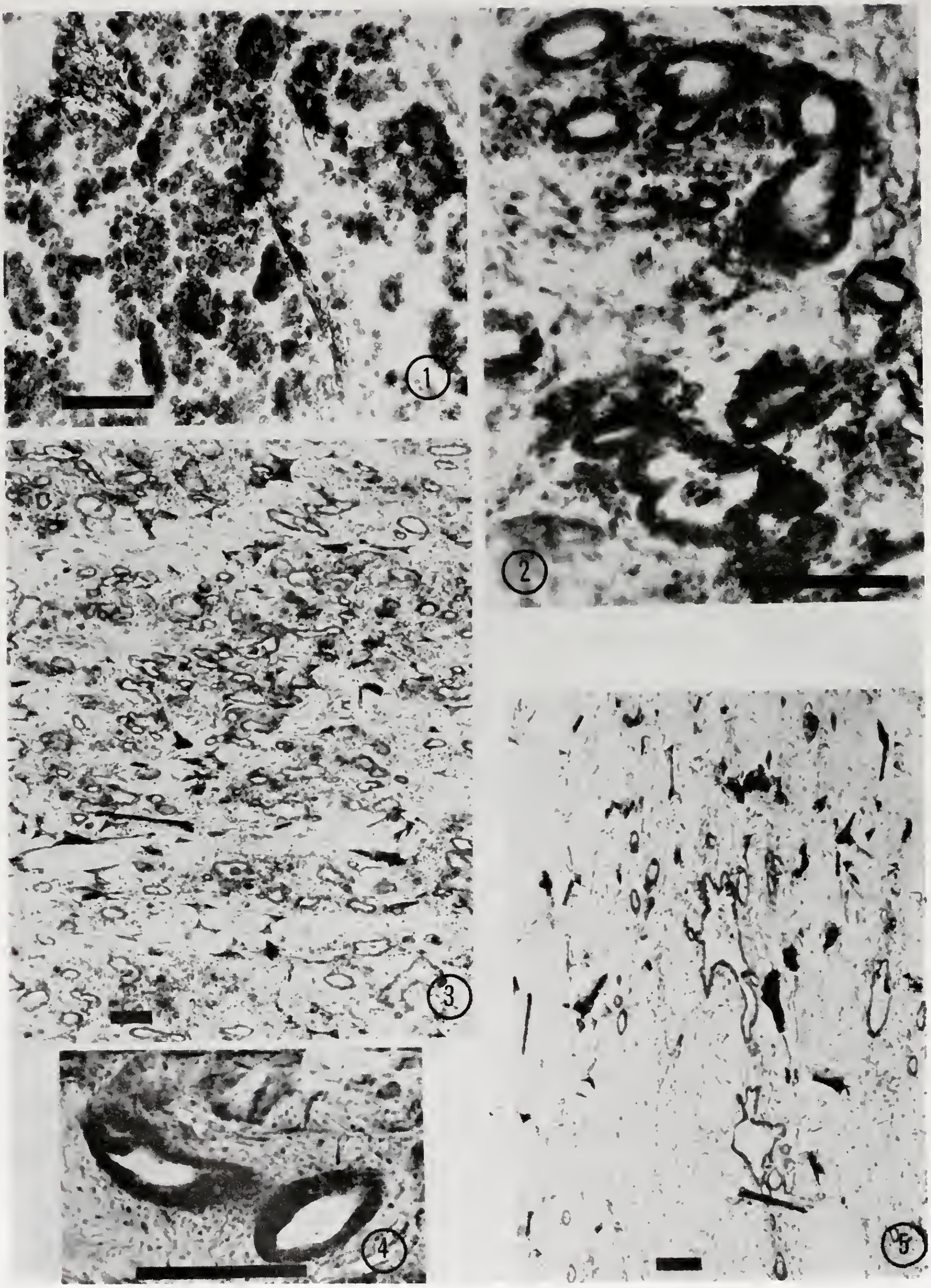

FIGURE 1. Control culture fixed immediately after preparation: Since the cells had been compacted by centrifugation and a certain time had elapsed until the culture was set up, cell aggregation is already under way. Scale bar equals 150 μ.

adverse manner. We are presently attempting to adapt the tissue plate technique so that post-embryonic cell viability would be increased, thus offering better opportunities for aggregation.

Lung on the sponge matrix

With single cells and cell-clump suspensions, extensive aggregation occurred with cells of 14-, 18-day embryos and, also, of newly hatched chicks. Histogenesis was excellent in plates of 14-day-old lung cells: bronchial tubules (Fig. 6) and cartilaginous rods were abundant. There was a quantitative decrease in reconstruction of tubules in plates of 18-day-old cells (Fig. 7), but no evident decrease in differentiation of cartilage. In plates from lung cells of hatched chicks, bronchial epithelium reconstructed tubules very rarely (Fig. 8); in several cases, capillaries with differentiated erythrocytes were found in these cultures (Fig. 10) and cartilagious rods or blocks were still occasionally present (Fig. 9).

The appearance of cells in the lung cultures suggested an insufficiency of a factor that would sustain the differentiated condition of the bronchial cells. After about a week of cultivation, epithelial cells in the tubules flattened, thus losing their typical morphology; after several more days bronchial epithelium degenerated and disintegrated while other types of cells still looked healthy and could survive much longer.

A single experiment with fully monodispersed cell suspensions of either 14- or 18-day embryos, confirmed the results described for the single cell, cell-clump suspensions. Extensive tissue plates formed which showed abundant bronchial tubules and nodules of cartilage. However, at the post-embryonic stages (immediately after hatching, 2-day and 4-day chicks) the proportion of erythrocytes in the sedimented material was very high and mortality of cells was considerable. As a result, solid tissue formed only as small aggregates, which in most cases were much too small to contain any tubular structure. Nevertheless, in a few instances a segment of a tubule lined with a healthy, bronchial epithelium was observed in cells from 2-day-old chicks.

Kidney and lung cells on Millipore filters

Cells of both organs reaggregated to some extent but did not show reorganization into tissue-specific structures (Fig. 11).

DISCUSSION

The present study is providing new information concerning age dependence of cell aggregation and reconstructive histogenesis; this has been possible because the problem has been reinvestigated with the help of the tissue plate technique

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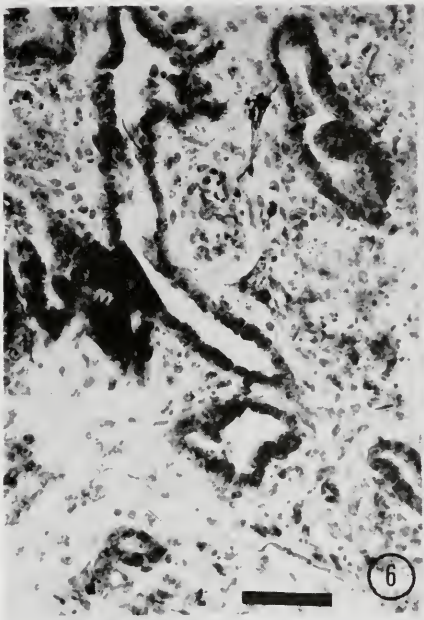
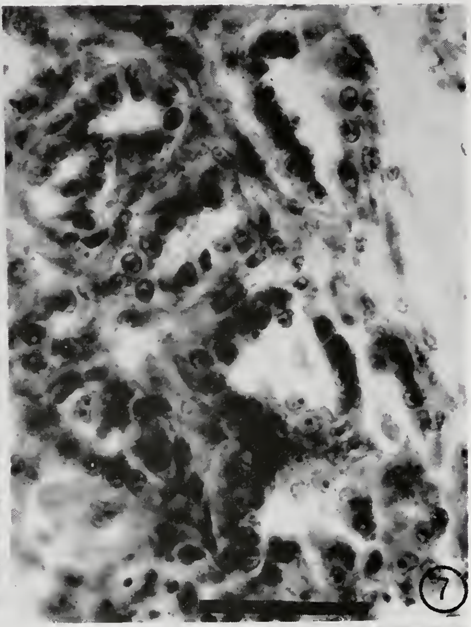
FIGURE 2. Tubules reconstructed in the tissue-plate prepared from 14-day-old embryonic kidney after 6 days of cutivation. Scale bar equals 300 μ.

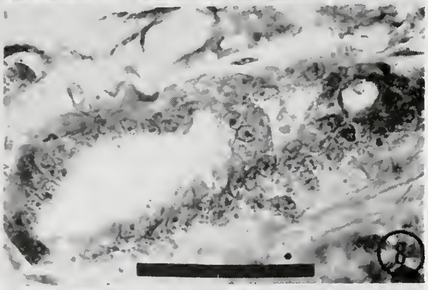
FIGURE 3. A small fragment (about 2 mm long) of a tissue-plate prepared from 18-day-old embryonic kidney after 6 days *in vitro*. Scale bar equals 70 μ.

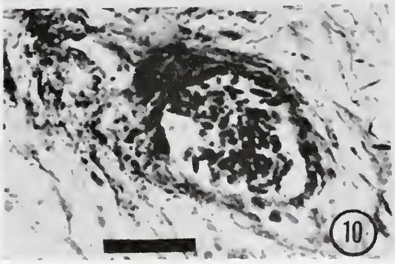
FIGURE 4. Nephric tubules in the tissue plate reconstructed by dispersed kidney cells of the hatched chick. Six days of cultivation. Scale bar equals 300 μ.

FIGURE 5. A culture similar to (3) but cultivated for 3 weeks. Scale bar equals 70 μ.









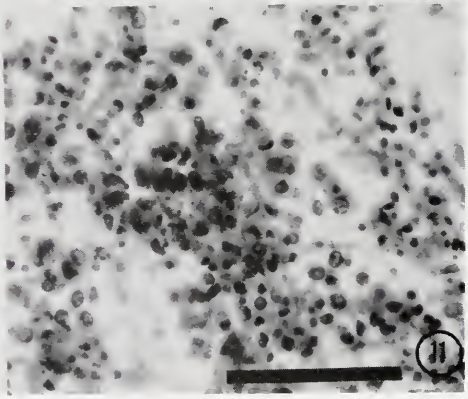


FIGURE 6. Bronchial tubules in the tissue-plate-culture prepared from 14-day-old embryonic lung. Six days of cultivation. Scale bar equals 150 μ.

(Ansevin and Lipps, 1973). This culture technique was developed by combining several older methods, such as cell aggregation and organ culture, with more recent techniques that utilize three-dimensional porous matrices as substrates for cells. By this means, the tissue plate culture technique overcomes several limitations inherent in each of the separate techniques. The application of a three-dimensional, porous matrix for the cells appears to promote their aggregation and subsequent histogenesis considerably more than do culture conditions which encourage formation of spherical aggregates. With the three-dimensional matrix, plate aggregates of relatively gigantic size are formed: about 10 mm in diameter and 0.3–0.5 mm in thickness. Spherical aggregates of embryonic cells obtained with previous methods, attained an average diameter close to 0.5 mm (Moscona, 1962) or 0.3 mm (Kuroda, 1968). Because considerably more cells are involved in formation of plate aggregates, their reconstructive histogenesis is superior to that of small spherical aggregates, a result consistent with the finding of Jones and Elsdale (1963) who determined that small size of aggregates was a limiting factor in their differentiation. In the present experiments, histogenesis occurred only in plate aggregates cultivated on three-dimensional porous substrates; cells cultured on Millipore filters aggregated but remained unorganized. We tentatively interpret this difference by assuming that convective circulation of medium is much less in three-dimensional substrates than around a thin Millipore filter and thus the products of cell interactions may remain at sufficient concentrations in the proximity of plate aggregates to promote their histogenesis.

The decline of aggregation and histogenesis with the age of the cell donor is much slower for plate aggregates cultivated on porous matrices than for spherical aggregates suspended in culture medium. In the experiments of Moscona (1962) and Kuroda (1968), cells from 18-day chick embryos essentially failed to aggregate; histogenesis was no longer present in aggregates formed by cells from 16-day embryos. In the conditions of the tissue plate culture, aggregation and histogenesis are excellent in cells of 18-day embryos; even at the immediately post-hatching stage, decline in aggregative capacities (of kidney cells) is not apparent and varying degrees of histogenesis (dependent on the type of tissue) are still accomplished. It was observed in the present experiments that cells of more advanced stages need longer time for reconstruction of tissue structures; this work is currently under progress.

To our knowledge, no comparable degree of aggregation or histogenesis was previously reported for cells of pre-hatching and post-embryonic chick stages or for similar developmental stages of mammals, although adult frog liver cells were shown to aggregate and exhibit histogenesis (Ansevin, 1964). The fact that fully mono-

FIGURE 7. Similar tubules in the plate-aggregate of 18-day-old embryonic chick. Four days of cultivation. Scale bar equals 150 μ.

FIGURE 8. A tubule in the tissue plate prepared from lung cells of hatched chick of cultivation. Six days of cultivation. Scale bar equals 300 μ.

FIGURE 9. Cartilage nodule differentiated in a week-old tissue plate prepared from lung cells of hatched chick. Twenty-one days of cultivation. Scale bar equals 150 μ.

FIGURE 10. A capillary-like structure with erythrocytes that is sometimes encountered in tissue-plate prepared from hatched chick lung cells. Seven days of cultivation. Scale bar equals 150 μ.

FIGURE 11. Lung-cells of 18-day-old chick embryo cultivated on Millipore filter for 4 days. Scale bar squals 300 μ.

dispersed cells from 4-day chicks can still occasionally form compact aggregates and, that these aggregates of post-embryonic cells, which are only semi-solid, appear to be highly contaminated with erythrocytes suggest that an intrinsic cell capacity for aggregation is not yet lost at young post-embryonic stages. Thus, it is possible that improved culture techniques would eliminate erythrocytes from cell suspensions and could decrease mortality of post-embryonic cells during preparation of the cultures, to permit further progress in the area of aggregation of post-embryonic cells. Since small size of aggregates in itself would be a factor hindering histogenesis (Elsdale and Jones, 1963), and, furthermore, since more solid and slightly larger aggregates of post-embryonic cells did show traces of histogenesis, it is likely that the low degree of histogenesis in the postembryonic cell cultures (following complete monodispersion) was again more of an expression of inadequate culture conditions than of inherent limitations in the post-embryonic cells. One may hope that this process could be evoked also in "older" cells by proper culture conditions.

This study also casts some light on the relationship between monodispersion of cells and their capacities to undergo subsequent aggregation and histogenesis. With cells of younger embryos (14 days) and even of those at 18 days of incubation differences between partly dispersed (including histologically structureless cell clumps besides single cells) and completely monodispersed cell suspensions were not obvious since both types of suspensions formed extensive tissue plates which were abundant in tubular and cystic structures. However, high mortality of fully monodispersed cells from newly hatched chicks was not evident in cultures which had been prepared with incompletely monodispersed suspensions; also, the former formed only small aggregates and showed only traces of histogenesis, while the latter were still able to aggregate into large tissue plates in which histological structures, although infrequent, were much better differentiated. This difference might be interpreted in general terms as a loss of a histogenetic factor from the cell surface during dispersion; a factor which would occur in larger quantities in embryonic than in post-embryonic cells. The presence of such factors has been determined in sponges (Humphreys, 1965) and strongly implied by Moscona's (1962) experiments with warm-blooded vertebrate cells.

We thank Mrs. Elga M. Lewis for assistance in histology. This work has been supported by grant No. PO1 HE 0925 of the National Institutes of Health.

Summary

Tissue plate cultures on a sponge matrix were prepared from kidney or lung cells of 14- or 18-day chick embryos, of newly hatched chicks, and of chicks 2 or 4 days old. Two types of suspension were prepared from these cells and used for the cultures: *suspension 1* contained single cells and cell clumps of varying size and degree of compactness, but free from organized histological structures; *suspension 2* contained single cells and 2-cell clumps exclusively. In the tissue plate cultures on the three-dimensional substrate both kidney and lung cells at both embryonic stages formed giant, plate-like aggregates from either type of suspension; histogenesis in these aggregates was excellent. Kidney cells of newly hatched

chicks were also able to reaggregate into extensive tissue plates from either type of suspension; reconstructed tubules were still present in these cultures although considerably less abundant. Lung cells from newly hatched chicks formed large tissue plates only from *suspension 1;* occasionally a bronchial tubule, a cartilage nodule or a capillary-like structure was found in the plates. *Suspension 2* of lung cells from newly hatched chicks resulted in formation of small aggregates with rare indications of a histogenetic process. The same result was true for both kidney and lung cells in *suspension 2* from 2- and 4-day-old chicks. The possibility is discussed that in these experiments technical reasons involved in preparation of the cultures were largely responsible for the apparent decline in aggregation and histogenesis of post-embryonic cells. In control experiments embryonic cells cultivated on Millipore filters ("two-dimensional," porous matrices) did not form extensive tissue plates or develop histological organization.

LITERATURE CITED

Ansevin, K. D., 1964. Aggregative and histoformative performance of adult frog liver cells *in vitro*. *J. Exp. Zool.*, **155**: 371–380.

Ansevin, K. D., and R. Buchsbaum, 1962. Capacity for histological reconstruction by mouse kidney cells in relation to age. *J. Gerontol.*, **17**: 130–137.

Ansevin, K. D., and B. V. Lipps, 1973. Tissue plate, a new technique for long term, organotypic culture. *In Vitro,* in press.

Grover, J. W., 1961. The relation between the embryonic age of dissociated chick lung cells and their capacity for reaggregation and histogenesis *in vitro*. *Exp. Cell Res.*, **24**: 171–173.

Humason, G. L., 1967. *Animal Tissue Techniques.* (2nd Ed.) Freeman, New York.

Humphreys, T., 1965. Cell surface components participating in aggregation: evidence for a new cell particulate. *Exp. Cell Res.* **40**: 539–43.

Jones, K. W., and T. R. Elsdale, 1963. The culture of small aggregates of amphibian embryonic cells *in vitro*. *J. Embryol. Exp. Morphol.* **11**: 135–154.

Kuroda, Y., 1968. Preparation of an aggregation-promoting supernatant from embryonic chick liver cells. *Exp. Cell Res.*, **49**: 626–637.

Moscona, A. A., 1961. Rotation-mediated histogenetic aggregation of dissociated cells. *Exp. Cell Res.* **22**: 455–475.

Moscona, A. A., 1962. Analysis of cell recombinations in experimental synthesis of tissues *in vitro*. *J. Cell. Comp. Physiol.*, **60**, Suppl. 1: 65–80.

Moscona, A. A., and H. Moscona, 1952. The dissociation and aggregation of cells from organ rudiments of the early chick embryo. *J. Anat.*, **86**: 287–301.

Reference: *Biol. Bull.*, **145**: 472–481. (December, 1973)

KINETIC STUDIES ON AMINO ACID UPTAKE AND PROTEIN SYNTHESIS IN LIVER OF TEMPERATURE ACCLIMATED TOADFISH

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Previous studies on the compensation of liver protein synthesis that occurs with temperature acclimation of toadfish have indicated that protein synthetic rate is controlled at the steps of polypeptide chain elongation and release (Haschemeyer, 1969a). Rate increases in cold-acclimated fish have been correlated with elevated levels of elongation factor I, the enzyme that promotes binding of aminoacyl-transfer RNA at the codon recognition site (Haschemeyer, 1969b). Chain elongation rate in liver is determined from the distribution of radioactivity between soluble completed proteins and total protein (including ribosome-bound growing chains) at short times (< 5 minutes) after injection of labelled amino acids. Use of the method, however, requires that uptake of the precursor and activation to aminoacyl tRNA be rapid compared with polypeptide chain assembly. We present here a new method for simultaneous measurement of L-amino acid uptake, activation, and incorporation into growing polypeptide chains by liver *in vivo*. Preliminary results have been reported (Haschemeyer and Persell, 1971).

MATERIALS AND METHODS

Animals

Adult toadfish, body weight 250 ± 30 g, about 90% male, were collected by the Supply Department at the Marine Biological Laboratory in June (1971 and 1972) and utilized in the following months of July and August. Fish were maintained in running sea water aquaria at 22° (normal laboratory sea water supply) or at 11° ± 1° using a Neslab seawater heat exchanger. The toadfish were fed killifish (*Fundulus heteroclitus*) to appetite until two days before experiment. When acclimation and experimental temperatures differed, 1 hour was allowed for adjustment to the new temperature before experiment.

Measurement of uptake and protein synthetic rates

The procedures for anesthesia and hepatic portal vein injection were described previously (Haschemeyer, 1969a). The injection solution contained 2 μCi of L-[U-^{14}C] amino acid mixture (0.01 μmole total) with 4 μCi of D-[1-^{3}H(N)] mannitol or [G-^{3}H]inulin in 1% NaCl, pH 7.2. Another series utilized 3 μCi of L-[U-^{3}H]leucine with 1–2 μCi of D-[1-^{14}C]leucine or [Carboxyl-^{14}C]-1-aminocyclopentane-1-carboxylic acid (cycloleucine). All isotopes were obtained from New England Nuclear Corp. In most experiments the air bladder of the fish was deflated by puncturing in order to relieve pressure against the hepatic portal vein that interfered with blood flow. The labelled compounds were injected into the portal vein in 0.1 ml saline carrier over a 3-second period. After an additional elapsed time of 3 seconds to 8 minutes the liver was rapidly excised and transferred to a Sorval Omnimixer can for homogenization as previously described (Haschemeyer, 1969a). Excision time (about 5 seconds) and time to homogenization (about 20 seconds) were noted for each animal. Immediately after liver excision, blood was collected in a heparinized syringe from the cut end of the hepatic veins leading to the heart. External pressure was applied to the heart region to facilitate drainage. After centrifugation the plasma was treated with cold 10% trichloroacetic acid (TCA). An aliquot of the supernatant fluid was analyzed to obtain the plasma free $^{14}C/^{3}H$ ratio. Radioactivity in the S (soluble protein) and T (total protein) fractions of the liver homogenate was determined on filter paper discs after fractionation according to Mathews, Oronsky and Haschemeyer (1973); washing of the discs included hot TCA for elimination of aminoacyl-tRNA (Haschemeyer 1969a). Assay of the combined incorporation into protein and aminoacyl-tRNA in these fractions was made by omitting the hot acid step, and the latter was obtained by difference. No radioactivity associated with the labelled marker substances (mannitol, inulin, D-leucine, cycloleucine) was found in the acid-precipitable fractions. The cold TCA supernatant fluid of an homogenate aliquot was analyzed to determine total recovery of ^{14}C and ^{3}H as free radioactivity in the liver. Counting was done in Aquasol (New England Nuclear) with a Packard scintillation spectrometer. Efficiency and overlap determination for the double label system was made with toluene internal standards. Results were normalized to an injection dose of 1 μCi for each component.

Determination of liver spaces

Water content was determined by drying either whole livers or blotted tips of lobes to constant weight in an oven at 90°. Determination of the time dependence of distribution of various substances in plasma and liver was done by injecting through a gill artery a saline solution containing 1–3 μCi of two of the substances listed in the previous section in ^{14}C-^{3}H pairs. After a given time for equilibration at 22° (5 minutes to 5 hours) blood samples were taken from the gills and from an internal vessel in heparinized syringes and the liver was rapidly excised and homogenized. Free radioactivities were determined for the cold TCA supernatant solution of plasma and liver homogenate aliquots in Aquasol. After correction for dilutions the recovery of each substance in liver (per g) was divided by its concentration in plasma (per ml) to obtain the volume in ml/g occupied by that substance in liver at the time of sampling.

RESULTS

At any time t after portal injection of a radioactive L-amino acid (A) the recovery in the excised liver will be the sum of amounts present in intracellular (i) and extracellular (e) spaces:

$$A_{\text{Liver}} = A_i + A_e \tag{1}$$

The intracellular contribution may be further subdivided as:

$$A_i = A_i^{Free} + A_i^{Protein} + A_i^{aa\text{-}tRNA} \qquad (2)$$

The quantities $A_i^{Protein}$, $A_i^{aa\text{-}tRNA}$ and the sum $[A_i^{Free} + A_e]$ which represents total free radioactivity in liver are determinable by use of the hot and cold acid extraction procedures described in materials and methods. $A_i^{aa\text{-}tRNA}$ refers to amino acid residues in aminoacyl transfer ribonucleic acid. These quantities thus yield A_{Liver}. The value of A_e must be determined by use of the marker data in order to obtain the desired A_i.

The recovery of the marker substance (B) in the liver will represent that amount present in extracellular space (B_e) plus any of the marker that has reached intracellular space by diffusion or other non-specific uptake processes (*e.g.*, endocytosis):

$$B_{Liver} = B_e + B_i^{diff} \cong B_e \qquad (3)$$

In the present study markers were used which showed negligible accumulation in liver during the short time course of the experiments. The term B_i^{diff} thus may be neglected without significantly affecting the final equations for the active transport of A.

Comparing equations (1) and (3) it is apparent that under circumstances of active accumulation of A by liver the total recovery A_{Liver} will exceed B_{Liver} by an amount proportional to the extent of specific or active transport (above that associated with non-specific processes). In order to eliminate the unknown A_e from equation (1) we use the measured value $(A/B)_{Plasma}$ obtained for plasma draining from the liver to approximate the ratio A_e/B_e in the extracellular space of liver. In this way A_e may be calculated directly from the experimental data, as follows:

$$A_e = (B_{Liver})\,(A/B)_{Plasma} \qquad (4)$$

A_i for each experimental time is then obtained from the measured total recovery of A in liver (A_{Liver}) by means of equation (1).

The time course for intracellular accumulation of radioactivity of the L-amino acid mixture at two experimental temperatures is presented in Figure 1. Fish of different acclimation groups were not distinguishable by this measurement, and data normalized to the average maximum uptake achieved for each experimental group are averaged together. The results clearly reveal a mechanism in liver for rapid intracellular uptake of L-amino acids injected into the hepatic portal vein. The system is essentially equilibrated within 1 minute at 24° and in about 2 minutes at 11°. After this time isotope ratios in plasma [$(A/B)_{Plasma}$] were in the range 0.2 to 0.5 compared with an injected ratio of 1.0, whereas uncorrected distribution ratios for liver [A_{Liver}/B_{Liver}] were as high as 6. At $t > 1$ minute (24°) the extracellular component A_e in liver calculated from equation (4) averaged $4 \pm 2\%$ of the injected dose. The distribution ratio for the free intracellular and extracellular pools [A_i^{Free}/A_e] reached values of about 15.

Recovery of D-mannitol (B_e) in liver showed an approximately exponential decline with time ($t_{\frac{1}{2}} \sim 20$ seconds at 24° and 35 seconds at 11°). By extrapolation of B_e to the level of the injected dose, a measure of flow time between the portal vein and the hepatic veins draining the liver (for the supine anesthetized fish) was obtained: 5–10 seconds at 24°; 10–15 seconds at 11°. Average recovery of

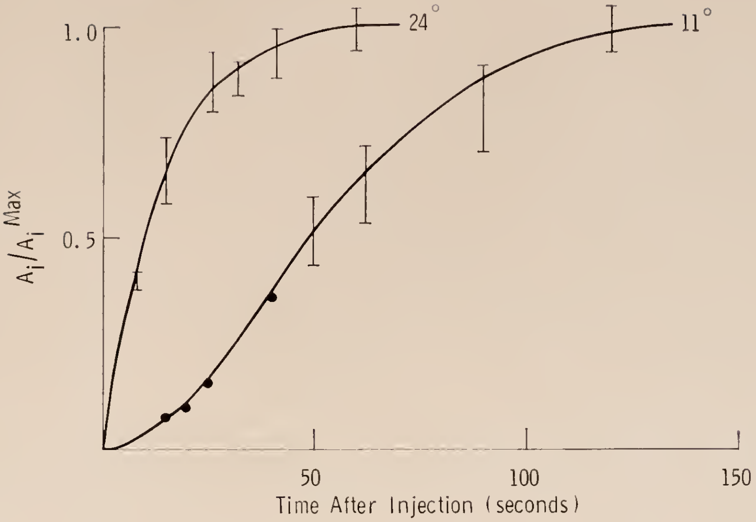

FIGURE 1. Kinetics of uptake of L-amino acids into intracellular space of toadfish liver following intraportal injection. Averaged data with standard error bars [based on $A_i(t)$] are shown for the ratio of uptake A_i at short incubation times to the maximum or equilibrium value A_i^{Max} obtained at $t > 1$ minute at 24° and $t > 2$ minutes at 11°.

D-mannitol (normalized to a 1 μCi injection) in liver in the time range of 1–2 minutes after injection at 24° was 0.18 ± .06. Other markers tested were not significantly different: D-leucine, 0.24 ± 0.04; inulin, 0.24; cycloleucine, 0.26 ± 0.04. None of these substances showed evidence of accumulation in liver during the short experimental periods used.

The accumulation process for A_i may be described by the simple differential equation:

$$dA_i/dt = k_a A_e - k_b A_i^{Free} \tag{5}$$

where the rate constant k_a for uptake into intracellular space will depend upon transport characteristics and, in this experimental system, on flow rate as well. The second term refers to efflux processes that remove A from the intracellular space. If protein turnover is neglected, efflux may be approximated by a first-order term in A_i^{Free}, the free radioactive pool. The rate constant k_b is dependent upon characteristics of the efflux system (*e.g.*, diffusion) that leads to loss of A_i from the intracellular space of liver as well as on flow rate.

Figure 2 presents a numerical solution of equation (5), *i.e.*, $(\Delta A_i/\Delta t)/A_e$ *vs.* A_i^{Free}/A_e, based upon the 24° data of Figure 1 and the corresponding averaged data for A_e and A_i^{Free} obtained in the same experimental series. The intercept and slope of this plot yield the following values for the parameters of equation (5): $k_a = 2.7$ minutes^{-1}; $k_b = 0.5$ minutes^{-1} (based on the earlier time points). From the value of k_a the half-time for uptake is found to be 15 seconds. At 11° an uptake half-time of 30 seconds may be calculated from the maximum rate of accumulation (Fig. 1) and the corresponding levels of A_e in this time range. The shape of the experimental curve at 11° (Fig. 1) clearly deviates from that pre-

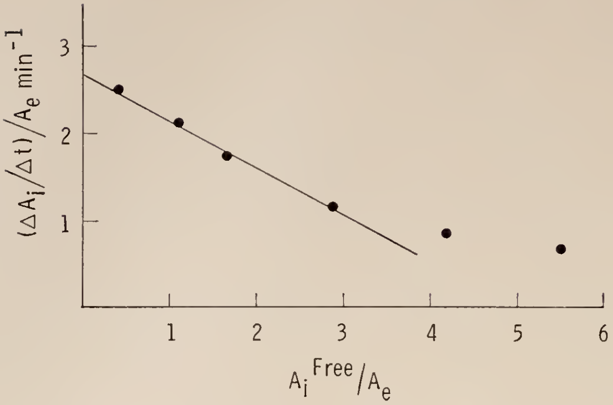

FIGURE 2. Graphical differentiation of the experimental data at 24° (Fig. 1) combined with A_i^{Max} (Table I) and $A_e(t)$. Points at higher values of A_i^{Free}/A_e are not included in evaluation of the constants of equation (5) on account of the large effect of experimental variation in this region. The linear portion yields uptake and efflux half-times of 15 seconds and 80 seconds, respectively.

dicted by equation (5). This is likely to be due to flow rate dependency and to the inadequacy of equation (5) for describing the system with the experimental data obtainable at short times after injection.

Amino acid uptake at equilibrium and protein synthetic parameters

At $t > 1$ minute at 24° and $t > 2$ minutes at 11° intracellular recovery of the L-amino acid radioactivity [A_i of equation (2)] appeared to have reached equilibrium within experimental error. Averaged results for A_i^{Max}, normalized to an injection dose of 1 μCi, are presented in Table I for fish of two temperature acclimation groups matched for body weight and nutrition. Total uptake was slightly higher for the 22°-acclimated group measured at 24°, but this may have been associated with differences in endogenous amino acid pools. Although both groups were starved for two days before experiment, the effect of starvation is likely to differ for fish at the two acclimation temperatures. In an earlier series 22°-accli-

TABLE I

Amino acid uptake levels and protein synthetic parameters for liver of toadfish in vivo *at 24° and 11°. Results are normalized to 1 μCi injection of the 15 L-amino acid mixture and presented with standard error and number of animals; body weight = 250 ± 30 g; liver weight = 6.8 ± 1.5 g*

Experimental temperature	Acclimation temperature	Experimental time range	A_i^{Max}	t_c (min)
24°	22°	1–5 min	0.76 ± 0.10 (6)	4.0 ± 0.4
	11°		0.63 ± 0.05 (6)	2.5 ± 0.3
11°	22°	2–8 min	0.56 ± 0.05 (6)	17 ± 2
	11°		0.61 ± 0.06 (6)	11.5 ± 1.5

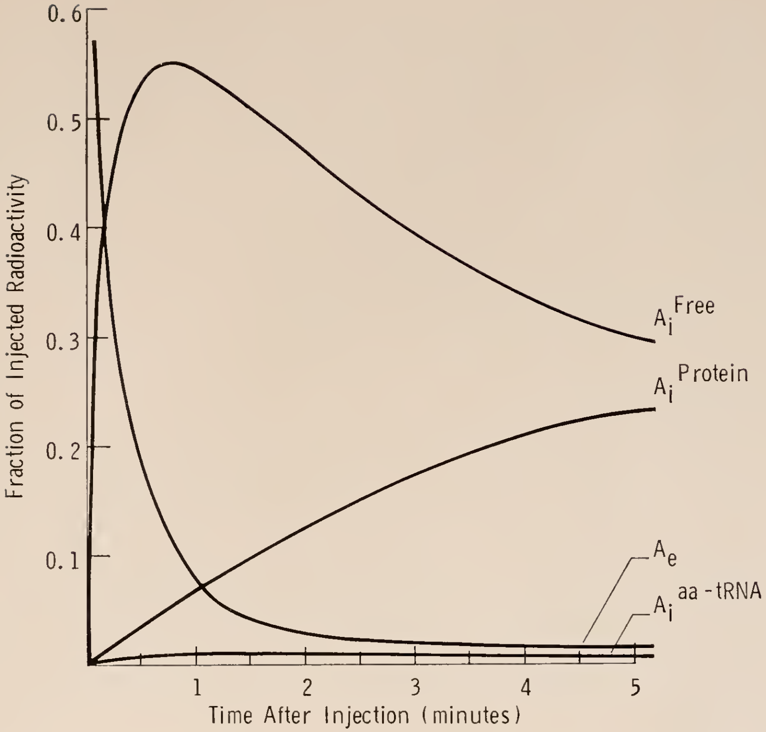

FIGURE 3. Time course in the distribution of radioactive L-amino acids among various liver pools following hepatic portal vein injection *in vivo* at 24° in toadfish acclimated for two weeks at 11°, A_i^{Free}—intracellular free (cold trichloroacetic acid soluble) amino acid pool; $A_i^{Protein}$—protein (hot acid insoluble) fraction; A_e—free amino acid pool attributed to extracellular space [from equation (4)]; $A_i^{aa\text{-}tRNA}$—aminoacyl transfer RNA.

mated fish that had been fed continuously showed a reduced average A_i^{Max} of 0.52 ± 0.08 at 24°.

Average polypeptide chain assembly time t_c (or the average time for one round of protein synthesis on all active ribosomes) was determined for each group from the distribution of radioactivity between growing (ribosome-bound) and completed chains as a function of incubation time up to t_c (Haschemeyer, 1969a). Values of t_c obtained from incorporation of the L-amino acid mixture and of L-leucine alone are given in the last column of Table I. The results at 24° indicate a slightly higher synthetic rate than previously reported at 22°. Part of the difference is due to temperature; other contributing factors are a shorter period of starvation before experiment and a change in methodology that reduced loss during centrifugation of membrane-bound protein. Cold acclimated fish showed more rapid protein synthesis (smaller t_c) at both experimental temperatures. Within each acclimation group Q_{10} for the process measured by t_c (polypeptide chain elongation and release) was about 3. Net incorporation into protein ($A_i^{Protein}$), though subject to more individual variation, roughly paralleled the

TABLE II

Determination of tissue spaces accessible to various substances in toadfish liver

Substance	Time after injection	Space* ml/g wet weight
Water	—	0.78 ± 0.02 (8)
D-mannitol, D-leucine, cycloleucine	1 to 3 hr	0.72 ± 0.03 (4)
D-mannitol	Zero (extrapolated)	0.31
Inulin	10 to 30 min	0.30 ± 0.03 (5)

* Including standard error and number of observations.

elongation rates as measured by t_c. This is in accord with results obtained at 22° (Haschemeyer, 1969a).

The distribution of labelling of the various liver pools that were measured directly ($A_i^{Protein}$ and $A_i^{aa\text{-}tRNA}$) or were calculated from the marker distribution (A_i^{Free} and A_e) is shown in Figure 3 by smoothed curves based on averaged data for the most active protein synthetic group. After the initial uptake period (0 to 1 minute) the principal measured change in the system is the transfer of labelled amino acid from the free pool to protein-bound form. Levels in the hot acid soluble fraction (aminoacyl-tRNA) amounted to about 0.5% of the injected dose, and remained essentially constant over experimental times of 0.25 to 5 minutes. The final level of incorporation into protein amounted to about 25% of the injected amino acid, and the average normalized incorporation rate was approximately 0.05 μCi/minute.

Space determinations in liver

Table II presents data for liver volume occupied by various substances, in relation to plasma concentration, at various times after arterial injection. Water content amounted to 78% of wet weight. After equilibration times of 1 to 3 hours diffusible substances such as mannitol occupied a liver space of 0.72 ml/g wet weight. A time series in the range of 10 to 60 minutes for mannitol distribution between plasma and liver was plotted in the form: log [1-mannitol space/H_2O space] *vs.* time to yield an extrapolated value at zero time of 0.31 ml/g, representing extracellular space occupied before permeation into intracellular spaces occurred. The half-time for equilibration obtained from the slope of the line was 20 minutes. The recovery of mannitol in liver, extrapolated to zero time, was 8.2% of the injected amount. Recovery after 30–60 minutes was 5%. Inulin space at equilibrium (10 to 30 minutes) was 0.30 ml/g; recovery, extrapolated to zero time, was 5.4% of the original dose. In contrast, recovery of ^{3}H-L-leucine injected by the same route averaged 19% at times from 5 to 60 minutes after injection.

DISCUSSION

A method is introduced here for determination of the time course and equilibrium level of uptake by liver of radioactively labeled L-amino acids supplied as a pulse via the hepatic portal vein. Analysis of the distribution of amino acids between intracellular and extracellular spaces in the time period of 5 seconds to 8 minutes after injection is based on concomitant measurements in liver and plasma of a marker substance of similar diffusivity that is not concentrated by liver. The time course of labelling of the liver aminoacyl transfer RNA and protein pools was also followed and average polypeptide chain assembly time determined.

The time course of uptake of a mixture of 15 L-amino acids supplied by the portal vein (Fig. 1) is shown to be rapid at both experimental temperatures (24° and 11°) in comparison with the time required for one round of protein synthesis (t_c) at these temperatures (4 minutes and 17 minutes, respectively, for 22°-acclimated fish). Equilibration times for the uptake process depended on experimental temperature but not on the acclimation temperature of the fish. Because the measurements are influenced by the rate of blood flow from the portal to hepatic veins, uptake half-times (15 seconds at 24° and 30 seconds at 11°) determined from equation (5) may principally reflect flow rate rather than transport parameters. Minimal values for flow time between injection site and drainage site were about 5–10 seconds at 24° and 10–15 seconds at 11°. The latter corresponds to a flow rate of 20 ml/min/kg body weight assuming the volume swept out is about half the liver inulin space. [Hepatic blood flow in dog is 30–35 ml/min/kg (Spurr and Dwyer, 1972)].

It is likely that most uptake occurs in the first pass of the injection fluid through the liver. Thereafter the system equilibrates by elimination of the extracellular components (A_e and the marker B_e) due to the influx of fresh blood from the portal vein and hepatic artery. The half-time for efflux of A_i from the liver is found to be 80 seconds at 24° [from k_b in equation (5)], compared with about 20 seconds for efflux of the marker B_e. This is not unexpected, since A_i is subject to continuous influx-efflux processes along the portal to hepatic vein pathway and to possible recycling of isotope re-entering the liver through the hepatic artery.

For the mixture of 15 L-amino acids injected at a total concentration of 0.1 mM (in 0.1 ml), intracellular uptake amounted to 60–70% of the injected quantity (Table I) at $t \geq 1$ minute at 24° or $t \geq 2$ minutes at 11°. Accumulation for L-leucine alone injected at 0.05 mM was about one-half as much. Following the initial uptake period, transfer of radioactivity from the free amino acid pool to protein-bound form is observed (Fig. 3). The distribution ratio between intracellular and extracellular spaces [A_i^{Free}/A_e] averaged about 15, based on recoveries in whole livers. When corrected for relative volumes of the two spaces [$v_i = 0.41$ ml/g, $v_e = 0.31$ ml/g (Table II)], the intracellular to extracellular concentration ratio is about 11.

These results are consistent with the operation of an active transport system in toadfish liver serving to accumulate L-amino acids against a concentration gradient. Comparison of the concentrations of the injection solutions with tissue amino acid levels [about 20 μmole/g for the amino acid mixture (Haschemeyer, 1968) and 0.2 μmole/g for leucine in similarly fed fish (Haschemeyer, unpublished)] indicates that transport probably was uphill throughout the measurement period. Selective accumulation of amino acids in liver relative to plasma has previously been observed (in mammals) after long periods of equilibration (see, *e.g.*, Harrison and Christensen, 1971). Data indicating active transport for several amino acids have been obtained in rat liver slices (Tews and Harper, 1969; Crawhall and Davis, 1971). The uptake of nonmetabolizable amino acids has been studied in perfused livers (Chambers, Georg and Bass, 1965; Mallette, Exton and Park, 1969).

The distribution ratios reported above do not include that portion of accumulated amino acid that has been transferred to aminoacyl-tRNA or protein at the time of measurement. Inclusion of this component would lead to higher uptake ratios. Also, no account has been taken of protein turnover in liver in the present study. Because of the short experimental times used it is doubtful that degradation processes would contribute significant radioactivity to the measured free amino acid pool. This factor complicates the interpretation of longer time experiments. The quantitative results for total A_i and the transfer of radioactivity from the free amino acid pool into protein (Fig. 3) indicate negligible loss of amino acids through chemical conversion. Such processes, *e.g.,* amino acid oxidation, may affect uptake results in long-term experiments (Fisher and Kerly, 1964).

Recovery of radioactivity in the aminoacyl tRNA pool amounted to about 0.5% of the injected amount for the amino acid mixture. The rapidity of equilibration of the pool (within 15–30 seconds at 24°) indicates that precursor activation kinetics will not cause serious error in the determination of polypeptide chain assembly times from incorporation data at $t > 1$ minute. Quantitative evaluation of the aminoacyl tRNA pool is possible from the data of Figure 3 and Table I for cold-acclimated fish measured at 24°. For this group the normalized incorporation rate into protein (0.05 μCi/min/liver) may be combined with the polypeptide chain synthesis rate [5.7 nmole/min/liver based on $t_c = 2.5$ min and ribosome concentration of 2.1 nmole/g (Haschemeyer, 1969a)] to obtain the average specific activity of the intracellular precursor pool. If the average polypeptide chain synthesized has 435 amino acid residues, this result turns out to be 0.020 μCi/μmole aminoacyl-tRNA. The normalized recovery of radioactivity in aminoacyl-tRNA then corresponds to an active pool of 37 nmole/g liver, representing an average of 18 molecules of aminoacyl tRNA per ribosome.

Overall protein synthesis in this system based on t_c for cold-acclimated fish at 24° is 2.5 mg/g liver/hour, the highest value obtained in toadfish liver to date. The synthesis rate for 22°-acclimated fish measured at 24° ($t_c = 4.0$ min) is 1.6 mg/g liver/hour, about $\frac{1}{5}$ of the mammalian rate calculated from t_c (1.16 min) and liver ribosome concentration in rat (Mathews, Oronsky and Haschemeyer, 1973). When temperature and ribosome concentration differences are taken into account, it would appear that the protein synthetic system of toadfish liver is comparable to the mammalian liver with respect to levels of substances involved in control of elongation rate.

The results herein provide further evidence of the value of poiklothermic vertebrates for the study of rate processes involved in liver protein metabolism *in vivo.* Body temperature may be varied both for examination of acute temperature effects and for study of adaptive changes associated with acclimation. Extension of the new methodology to determine transport parameters and hormonal influences on these kinetic systems is in progress.

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SUMMARY

1. A new method is described for measurement of intracellular uptake of amino acids by liver in toadfish (*Opsanus tau*) *in vivo* with simultaneous determination of incorporation into aminoacyl transfer RNA and of polypeptide chain assembly time, a kinetic parameter for protein synthesis.

2. Mixed L-amino acids or L-leucine supplied by a pulse injection into the hepatic portal vein are strongly concentrated by liver (up to 75% of dose) compared with D-amino acid or mannitol markers. Uptake occurs against an apparent concentration gradient with halftimes of 15 seconds at 24° and 30 seconds at 11°.

3. The uptake of pulse-injected L-amino acids into intracellular space of liver and the equilibration of the aminoacyl-tRNA pool are rapid compared with the kinetics of polypeptide chain assembly and thus do not interfere with assembly time determination.

4. Polypeptide chain assembly times were 4.0 and 2.5 minutes for warm- and cold-acclimated fish, respectively, at 24° and were 17 and 11.5 minutes for the same groups measured at 11° The Q_{10} (for acute temperature change) was about 3 for both acclimation groups.

LITERATURE CITED

CHAMBERS, J. W., R. H. GEORG AND A. D. BASS, 1965. Effect of hydrocortisone and insulin on uptake of α-aminoisobutyric acid by isolated perfused rat liver. *Mol. Pharmacol.*, **1**: 66–76.

CRAWHALL, J. C., AND M. G. DAVIS, 1971. Further studies of the transport of amino acids in rat liver slices. *Biochim. Biophys. Acta*, **225**: 326–334.

FISHER, M. M., AND M. KERLY, 1964. Amino acid metabolism in the perfused rat liver. *J. Physiol.*, **174**: 273–294.

HARRISON, L. I., AND H. N. CHRISTENSEN, 1971. Evidence for a hepatic transport system not responsive to glucagon or theophylline. *Biochem. Biophys. Res. Commun.*, **43**: 119–125.

HASCHEMEYER, A. E. V., 1968. Compensation of liver protein synthesis in temperature-acclimated toadfish, *Opsanus tau*. *Biol. Bull.*, **135**: 130–140.

HASCHEMEYER, A. E. V., 1969a. Rates of polypeptide chain assembly in liver *in vivo*: relation to the mechanism of temperature acclimation in *Opsanus tau*. *Proc. Nat. Acad. Sci.*, **62**: 128–135.

HASCHEMEYER, A. E. V., 1969b. Studies on the control of protein synthesis in low temperature acclimation. *Comp. Biochem. Physiol.*, **28**: 535–552.

HASCHEMEYER, A. E. V., AND R. PERSELL, 1971. Amino acid transport and plasma protein synthesis in toadfish liver *in vivo*. *Biol. Bull.*, **141**: 389.

MALLETTE, L. E., J. H. EXTON AND C. R. PARK, 1969. Effects of glucagon on amino acid transport and utilization in the perfused rat liver. *J. Biol. Chem.*, **244**: 5724–5728.

MATHEWS, R. W., A. ORONSKY AND A. E. V. HASCHEMEYER, 1973. Effect of thyroid hormone on polypeptide chain assembly kinetics in liver protein synthesis *in vivo*. *J. Biol. Chem.*, **248**: 1329–1333.

SPURR, G. B., AND N. J. DWYER, 1972. Hepatic blood flow and indocyanine green disappearance in hyperthermia and endogenous fever. *J. Appl. Physiol.*, **32**: 362–368.

TEWS, J. K., AND A. E. HARPER, 1969. Transport of nonmetabolizable amino acids in rat liver slices. *Biochim. Biophys. Acta*, **183**: 601–610.

Reference: *Biol. Bull.*, **145**: 482–491. (December, 1973)

DISTRIBUTION AND ABUNDANCE OF *PINNOTHERES MACULATUS* SAY IN BOGUE SOUND, NORTH CAROLINA

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The pea crab, *Pinnotheres maculatus* Say, is a symbiont found in the mantle cavity of many bivalve molluscs. The bay scallop, *Argopecten irradians concentricus* (Say), is its most common host in the inland waters of North Carolina.

Observations on the biology of *P. maculatus* are limited to a list of hosts in which the crab are found (Williams, 1965), a description of its larval stages (Costlow and Bookhout, 1966), post-larval life cycle (Pearce, 1964), studies concerning attraction of adult crabs to hosts (Sastry and Menzel, 1962; Yeater, 1965), and effect of light intensity and temperature on swimming velocity of zoeae (Welsh, 1932). Kruczynski (1972) demonstrated that adult female crabs cause a decrease in growth and dry weight of bay scallops in Bogue Sound, North Carolina.

The object of this study was to determine how the crabs are distributed in scallops in Bogue Sound, and to investigate factors which may control their distribution and abundance.

Materials and Methods

Study area

Bogue Sound is a shallow lagoon approximately 37 km long and 3.7 km wide at its widest point, averaging about 1 m in depth at low tide (Fig. 1). The Intercoastal Waterway closely parallels its north shore and is 6 m deep. Ocean water is supplied through two inlets, one at each end. Fresh water is received from creeks and runoff along its north shore as well as from a river at each end.

Benthic grass (*Zostera* and *Halodule*) is contagiously distributed throughout the sound except in the inland waterway. Scallops are found throughout the sound where grass occurs or was recently abundant.

Zoeae distribution and abundance

Monthly plankton tows were made at eight areas along the length of Bogue Sound from June 1969 to June 1970 (Fig. 1, Sites 1, 3, 4, 7, 8, 10, 12, 13). No plankton samples were taken in October, February and May. Tows were made at night, pulling a plankton net behind a boat moving at "trolling speed" for ten minutes, thus semi-quantifying samples. Surface salinity and temperature were measured while towing. Plankton samples were preserved in formalin and approximate volume of plankton for each tow measured in a graduated cylinder after the manner of Wickstead (1965). An aliquot was examined under a dissecting microscope and zoeae of *P. maculatus* removed and counted. An estimate

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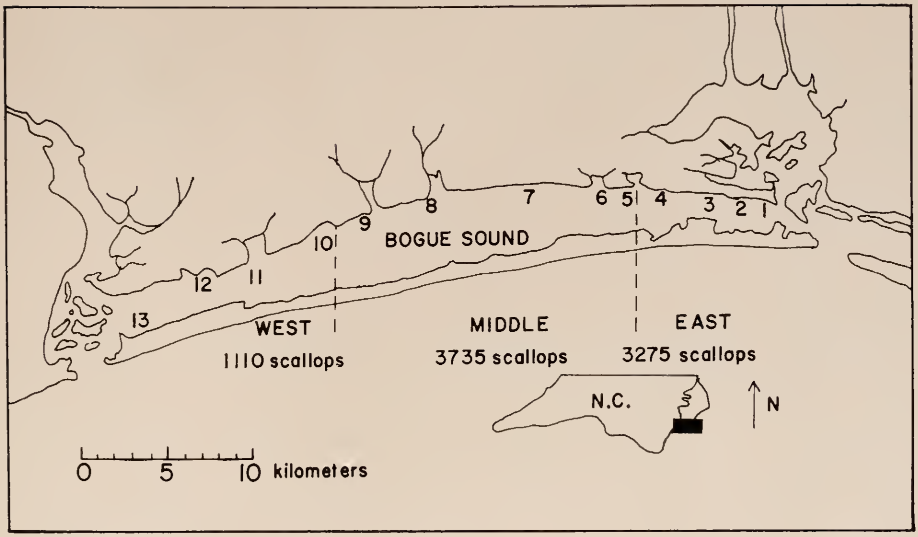

FIGURE 1. Collection sites in Bogue Sound, North Carolina. Sites of plankton collections and salinity measurements are: 1, 3, 4, 7, 8, 10, 12, 13. Sites of quadrat sampling are: 2, 3, 5, 6, 7, 8, 9, 12. Sites of gridded scallop beds are: 3, 4, 7, 8, 9, 10, 12, 13.

of total number of zoeae per tow was made from the ratio picked volume to total volume of sample.

Distribution and abundance of post-larval P. maculatus

Post-larval crabs occur in scallops, so seasonal abundance and distribution of these stages was studied by collecting scallops from various areas in the sound from 1968 to 1970. Scallops were collected by hand or with a small dredge from shallow-water grass beds. Scallops were taken to the laboratory, measured, opened and inspected for crabs. Crabs were measured and life-cycle stage determined. Seminal receptacles of all females were dissected and smear mounts examined for presence of sperm.

Scallop distribution and abundance

The possibility that crab distribution may be controlled by the abundance of scallops arose early in this study and two methods were used to test this theory.

Transects. Eight locations (Fig. 1, Sites 2, 3, 5, 6, 7, 8, 9, 12) where scallops were known to occur were chosen and a series of transects made at each site. All transects ran from low tide mark on the north shore to waist deep water. One-square-meter quadrats were cleared every 5 m and scallops counted. Some scallops were taken back to the laboratory for further examination and crabs were noted. Presence or absence of a grass bottom was recorded for each quadrat.

Grids. To further assess scallop density and total numbers, eight scallop beds were gridded and compared (Fig. 1, Sites 3, 4, 7, 8, 9, 10, 12, 13). These beds

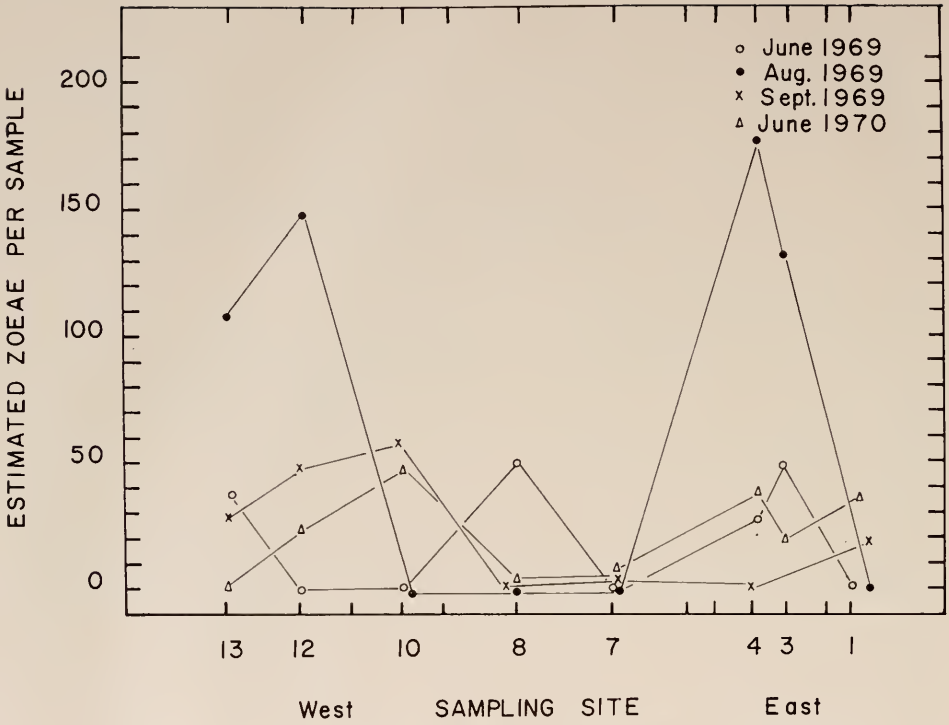

FIGURE 2. Abundance of zoeae of *Pinnotheres maculatus* in Bogue Sound in selected months of 1969 and 1970.

were chosen because scallops were found to occur in good numbers at each site. A portion of each bed was divided into a grid by driving stakes every 25 m along a square. Each staked area enclosed 2500 m^2. At time of sampling, total grass cover of the gridded area was sketched. A random numbers table was used to fix the center of each sample arc. Samples were made only when this center fell on a grassy area. A circle with a 5 m radius (78 m^2), or a half circle (39 m^2) was cleared with a scallop rake or by hand around the randomly chosen grassy center. Scallops collected were taken to the laboratory, counted, measured and opened. Crabs were noted. An estimate of the total number of scallops for each gridded area was made by multiplying mean density of scallops by the area covered by grass.

RESULTS

Zoeae distribution and abundance

Zoeae were found from April through September and were most abundant in the east and west ends of the sound in August (Fig. 2). Zoeae were not found from November to March when surface temperature was below 12° C but began to appear in April when surface temperature was above 12° C. Zoeae were scarce in the middle areas of the sound except for one sample collected on 16 June 1969 (Fig. 2, Area 8). No adult crabs were naturally found in this area, however, two days prior to the tow a wire box which contained 10 scallops, each with an ovigerous female crab, was placed on the grass bed where the plankton tow was made. This was done to determine whether adult female crabs could survive in this area. They did survive and it is probably their zoeae which were captured in the sample since few zoeae were later found after removing this box of infected scallops.

Surface salinity was generally higher near the inlets of Bogue Sound and decreased toward the center (Fig. 3).

Post-larval P. maculatus

Inspection of 8120 scallops collected during all months from 1968 to 1971 revealed the post-larval life cycle of *P. maculatus* in Bogue Sound. All stages were more abundant near inlets and absent from center areas of the sound.

Pre-swarming crabs. The first crab instars were found in scallops abundantly in October and November near the inlets (Sites 1, 2, 3, 4, 5, 6, 11, 12, 13) and multiple infections were common. These crabs remained in scallops for several molts and ranged in carapace width from 1 to 6 mm. One hundred fifty-one pre-swarming crabs (60 female, 85 male) were collected. No females in this stage contained sperm in the seminal receptacles.

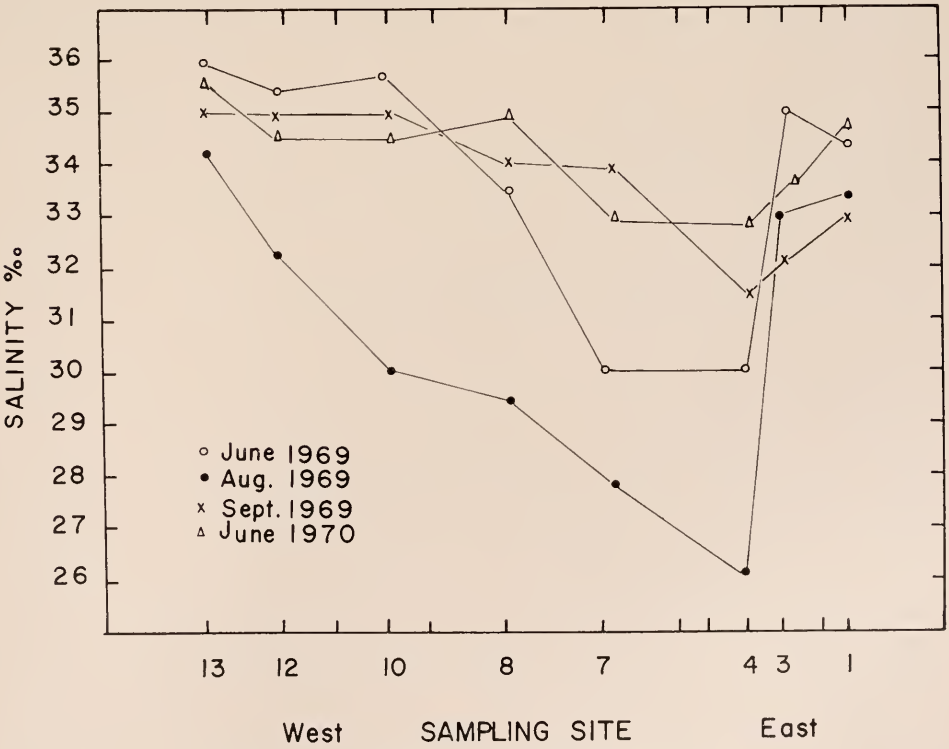

FIGURE 3. Surface salinity at plankton stations.

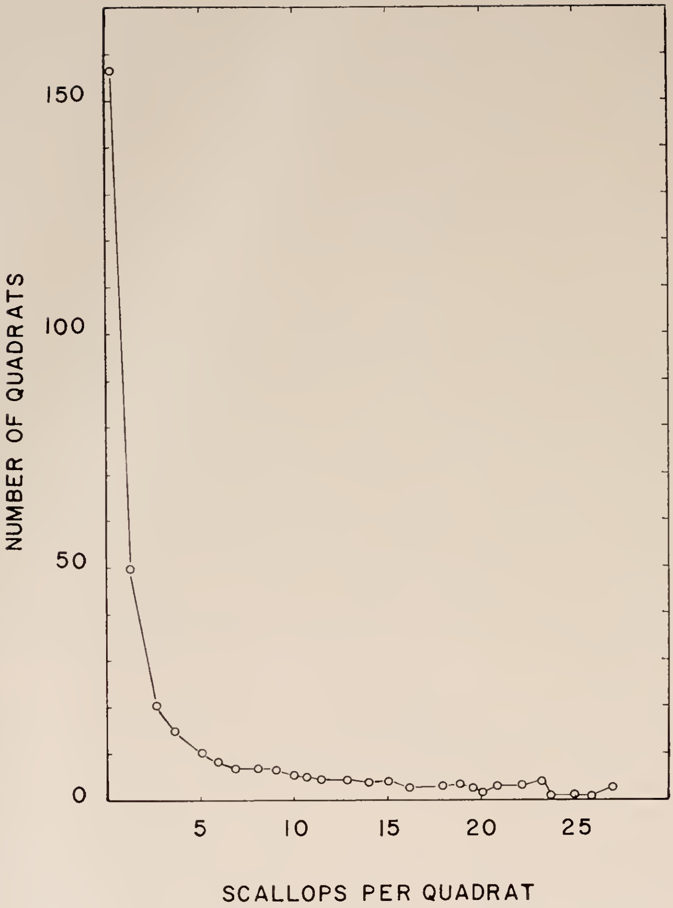

FIGURE 4. Abundance of scallops in quadrats along transect lines.

Swarming crabs. Swarming crabs are specialized for existence in open water and possess swimming hairs on the third, fourth and fifth legs, and are flattened to facilitate entrance into scallops. Swarmers were found in scallops near inlets (Sites 1, 2, 3, 4, 5, 6, 12, 13) in the fall and copulation took place in the open water at this time (this was not observed).` Three hundred forty-five (145 female, 200 male) swarmers were collected and they varied in carapace width from 3 to 10 mm. Seminal receptacles of 65% of the swarming females collected in November were found to contain sperm.

Overwintering occurred in either pre-swarming or swarming stage and both stages were found in scallops in March and April. Since some swarmers were found in spring which were not inseminated, it is possible that copulatory swarming may continue in early spring.

Post-swarming crabs. After swarming, males enter scallops and end their life cycle in the swarming stage. They usually accompanied a female crab in scallops throughout the summer. Females molted into an intermediate stage(s) in which the abdomen was not yet fully developed to carry eggs. Thirty-two intermediates were collected from June through November which ranged in size from 5 to 8 mm in carapace width; all contained sperm. One hundred and sixty-seven ovigerous females were collected from scallops in June through December and ranged from 7 to 14 mm carapace width; all contained sperm. During the summer months only post-swarming crabs were found and only at stations very close to the inlets of the sound (Sites 1, 2, 3, 4, 13). No scallops were found which contained more than one post-swarming female crab.

Seasonal abundance. Seasonal abundance of post-larval stages was followed for various scallop beds. Monthly observations for two years at one area (Site 3), where crabs were most abundant, are summarized below. Ten per cent of 420 scallops collected in June through August contained ovigerous female crabs. Only 6 males were collected and all occurred in a scallop containing an adult female crab. Four hundred and six scallops were collected from September through November and 4% contained ovigerous females, 57% pre-swarming and 42% swarming crabs. In December through February most scallops (about 60 mm shell height) are removed by commercial harvesting, so only 109 scallops were collected during this quarter. No adult females were found, 10% contained pre-swarming crabs, and 6% contained swarming crabs. Scallops collected in March, April and May (210) were predominantly young scallops (25 mm shell height) and contained few pre-swarmers (3%) and swarmers (9%). By June, ovigerous females were found in 9% of scallops (40 mm shell height).

Scallop distribution and abundance

Three hundred thirty-three quadrats were sampled along transects at the eight areas. Frequency of scallops per quadrat fits the negative binomial distribution ($P < 0.05$) which describes positive contagion (FIG. 4). There is a positive correlation (Chi Square $P < 0.05$) of quadrats containing scallops and presence of grass (Table I). Mean scallop density per m^2 was: 2.6, 2.9, 4.3, 3.6, 4.4, 2.5, 1.7, and 2.3; the largest densities found in middle sound, Sites 5, 6 and 7.

Table II summarizes data from gridded areas. Mean density and estimated total numbers were higher for middle sound sites.

DISCUSSION

On the basis of crab distribution in all scallops collected, Bogue Sound can be divided into three areas: East, Middle and West (Fig. 1). Mature female crabs were found only in scallops from east and west sound. All other crab stages were more abundant in these areas, but occurred in marginal middle collections in reduced numbers. Why the crabs are not found in scallops in the middle sound remains an enigma. Scallop density was highest in the mid-sound beds studied by transecting and gridding. Mature female crabs were never found to be really abundant even near the inlets (10%), the most favored areas, so it is unlikely that abundance of hosts controls numbers or distribution of crabs.

Few other studies mention abundance of *P. maculatus* in hosts. Sastry and Menzel (1962) found bay scallops infected with adult females to vary from 13 to

TABLE I

Two-by-two contingency table of quadrat samples along transects at eight sites in Bogue Sound

	Grass	No grass	
Scallops	167	10	177
No scallops	37	119	136
	204	129	333 total quadrats

36% of the total population in Alligator Harbor, Florida. Yeater (1965) found monthly incidence of bay scallops with adult female crabs to vary between 0 and 40% in the same harbor. No females were found in winter months during his study. Pearce (1964) found 97.6% of 1820 *Mytilus edulis* collected at Quicks Hole, Massachusetts, from 5 July 1963 to 5 August 1964, infected with some stage of *P. maculatus.*

I believe the low percentage of adult crab infection in Bogue Sound may be attributed to the commercial scallop fishery which keeps the percentage of scallops infected with post-swarming crabs low. Overwintering crabs are found in great numbers in commercially harvestable scallops. At shucking houses the scallop muscle is saved and shell and viscera discarded near the edge of the sound. Thus, large numbers of pre-adult crabs are killed each winter. However, this theory does not explain why surviving crabs are found only near the inlets of the sound.

Several other cases of geographic variation in host preference and host infection by pinnotherid crabs have been found by Williams and Needham (1939), and Gray (1961). MacGinitie and MacGinitie (1949) found that *Pinnixa eburna* Wells infected tubes of *Arenicola* worms which occurred in sand and were never found with worms in adjacent mud flats. Pearce (1966) found a correlation between water depth at which *Modiolus modiolus* were found and per cent infection by *Fabia subquadrata;* 0 to 20 m depth was optimum for the crab. Crabs taken from mussels in deeper water tended to be smaller. Depth of host animal was also found to be a factor controlling distribution of *Pinnotheres pisum* in mussels (Houghton, 1963; Seed, 1969) and *Pinnotheres ostreum* in oysters (Beach, 1969).

TABLE II

Grass cover, sampling area, scallop density and estimated total scallops of gridded areas

Area	Estimated grass cover-m^2	Number samples taken	Total area cleared-m^2	Mean density per m^2	Estimated total no.
3	1525	3	234	0.6	930
4	1562	3	156	1.4	2250
7	1000	3	156	3.9	3890
8	1050	3	195	2.2	2352
9	900	2	156	2.3	2097
10	1090	2	156	1.2	1305
12	1075	1	78	2.1	2300
13	638	2	117	1.2	733

Per cent infection was observed to be greatest below low tide line, and Beach theorized that the longer the submergence of hosts, the more opportunity for invasion.

Goodbody (1960) suggested that abbreviated development of zoeal stages in *Pinnotheres moseri* Rathbun may be an adaptation preventing larvae from dispersing too far away from concentrations of its host population. No abbreviated development is known to occur in *P. maculatus*. Assuming that tidal currents sweep *P. maculatus* larvae into the middle of Bogue Sound, the fact that adults are not found there suggests that the area does not satisfy the species requirements. Hosts are plentiful, no competition with other animals is known, and there is no evidence of increased predation; therefore some physical or chemical requirements may be lacking. That adults can survive in this area, at least for a short time, has been established, so the limiting factor probably acts on a preadult stage. Surface salinity was the only environmental variable measured in this study. It is possible that pronounced salinity gradients could be produced during times of heavy rains and land runoff.

The effect of salinity on the distribution of the closely allied *Pinnotheres ostreum* is well understood. Beach (1969) found that salinity may exert a marked effect on larval stages of *P. ostreum* because embryonic development is not possible below 15 ‰, although adults could survive lower salinities. Nagabhushanam (1965) found salinity from 6 to 7‰ lethal for adult *P. ostreum* within 24 hours. Stauber (1945) observed death of "hard stage" *P. ostreum* during periods of low salinities whereas female crabs in later stages of development were able to survive these salinities. Flower and McDermott (1952) found a graduated percentage of oysters with crabs along the length of Delaware Bay. Oysters from the lower, more saline waters were infected more than oysters from the upper reaches of the bay. Pinschmidt (1963) described a decrease of *P. ostreum* larvae in upper reaches of the Newport River North Carolina estuary. His findings indicate a net downstream displacement of crab larve because of tides. First stage (invasive) crabs were more numerous in higher salinity waters of the lower estuary.

Read (1968) studied salinity as a potential limiting factor in the distribution of both *P. ostreum* and *P. maculatus*, and determined that *P. maculatus* exhibits good survival at 25° C in only 20 and 30‰ water, and concluded that higher salinities were conducive to its survival. Read also found that *P. maculatus* was a weak osmotic regulator and its body cells were intolerant of low osmotic concentrations; however, because the bay scallop is found in 20 to 38‰, he concluded that *P. maculatus* will be found in all areas inhabited by the scallop. This is clearly not so in Bogue Sound. Walker (1969) showed that *Halicarcinus lacustris* (Chilton), a brackish water crab, can tolerate a wide range of salinity in the laboratory, yet normally occur in a narrow range in Australian inland waters.

Other factors, such as tidal current, could possibly control distribution of the crab in Bogue Sound scallops. The position of certain species can be maintained by selectively using certain stages of tidal current (Verwey, 1960; Stieve, 1961; Hughes, 1969). Such behavior could keep larval or swarming *P. maculatus* near east and west Bogue Sound. I visualize the control of distribution of *P. maculatus* in Bogue Sound as follows: larvae are swept to all reaches of the sound by tidal currents but survive best near inlets. Scallops are invaded in great numbers in fall and early winter. Heavy rains at this time reinforce the salinity gradient in Bogue Sound. Many pre-swarmers and swarmers are lost because of scallop harvesting. Swarmers receive an external cue and begin a migration on ebb tides becoming concentrated near inlets, or use tides to maintain near-inlet position. Copulation takes place and inseminated females seek a definitive host in the area where copulation occurs.

Studies of the distribution of *P. maculatus* in *M. edulis* in a more northern estuary with a salinity gradient paralleling that in Bogue Sound would be interesting because the mussel has a wide salinity tolerance (10 to 30‰).

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SUMMARY

1. All stages of *Pinnotheres maculatus* were more abundant near the inlets of Bogue Sound, North Carolina.

2. Distribution and abundance of adult crabs did not conform to distribution of scallops, since scallops were numerous throughout the sound.

3. It is possible that crab distribution is controlled by a physical or chemical gradient such as salinity.

LITERATURE CITED

BEACH, N. W., 1969. The oyster crab, *Pinnotheres ostreum* Say, in the vicinity of Beaufort, North Carolina. *Crustaceana,* **17**: 187–199.

COSTLOW, J. D., AND C. G. BOOKHOUT, 1966. Larval stages of the crab, *Pinnotheres maculatus,* under laboratory conditions. *Chesapeake Sci.,* **7**: 157–163.

FLOWER, F. B., AND J. J. MCDERMOTT, 1952. Observations on the occurrence of the oyster crab, *Pinnotheres ostreum,* as related to oyster damage in Delaware Bay. *Nat. Shell. Ass., Conv. Addresses,* **1952**: 44–50.

GOODBODY, I., 1960. Abbreviated development of a Pinnotherid crab. *Nature,* **185**: 704–705.

GRAY, I. E., 1961. Changes in abundance of the commensal crabs of *Chaetopterus. Biol. Bull.* **120**: 353–359.

HOUGHTON, D., 1963. The relationship between tidal level and occurrence of *Pinnotheres pisum* in *Mytilus edulis. J. Anim. Ecol.,* **32**: 253–257.

HUGHES, D. A., 1969. Responses to salinity change as a tidal transport mechanism of pink shrimp, *Penaeus duorarum. Biol. Bull.,* **136**: 43–53.

KRUCZYNSKI, W. L., 1972. The effect of the pea crab, *Pinnotheres maculatus* Say, on growth of the bay scallop, *Argopecten irradians concentricus* (Say). *Chesapeake Sci.,* **13**: 218–220.

MACGINITIE, G. E., AND N. MACGINITIE, 1949. *Natural History of Marine Animals.* McGraw Hill Book Co., New York, 473 pp.

NAGABHUSHANAM, R., 1965. Tolerance of the pea crab, *Pinnotheres ostreum,* to waters of low salinity. *Curr. Sci.,* **34**: 22–23.

PEARCE, J. B., 1964. On reproduction in *Pinnotheres maculatus. Biol. Bull.,* **127**: 384.

PEARCE, J. B., 1966. The biology of the mussel crab, *Fabia subquadrata,* from the waters of the San Juan archipelago, Washington. *Pacific Sci.,* **20**: 3–35.

PINSCHMIDT, W. C., JR., 1963. Distribution of crab larvae in relation to some environmental conditions in the Newport River estuary, North Carolina. *Ph.D. dissertation, Duke University,* Durham, 120 pp.

READ, D. E., JR., 1968. Salinity tolerance and osmoregulation of the pea crabs *Pinnotheres ostreum* Say and *Pinnotheres maculatus* Say. *Masters thesis, Duke University,* Durham, 61 pp.

SASTRY, A. N., AND W. MENZEL, 1962. Influence of hosts on the behavior of the commensal crab *Pinnotheres maculatus* Say. *Biol. Bull.,* **123**: 388–395.

SEED, R., 1969. The incidence of the pea crab, *Pinnotheres pisum* in the two types of *Mytilus* (Mollusca: Bivalvia) from Padstow, south-west England. *J. Zool.,* **158**: 413–420.

STAUBER, L. A., 1945. *Pinnotheres ostreum* parasitic on the American oyster *Ostrea virginica. Biol. Bull.,* **88**: 269–291.

STEIVE, H., 1961. Sinnes physiologische Betrachtungen uber die Orientierung der Meerestiere bei ihren Wanderungen. *Helgolaender Wiss. Meeresunters.,* **8**: 153–166.

VERWEY, J., 1960. Uber die Orientierung wandernder Meerestiere. *Helgolender Wiss. Meeresunters.,* **7**: 51–58.

WALKER, K., 1969. The ecology and distribution of *Halecarcinus lacustris* (Brachyura: Hymenosomatidae) in Australian inland waters. *Aust. J. Mar. Freshwater Res.,* **20**: 163–173.

WELSH, J. H., 1932. Temperature and light as factors influencing the rate of swimming of larvae of the mussel crab, *Pinnotheres maculatus. Biol. Bull.,* **63**: 310–326.

WICKSTEAD, J. H., 1965. *An Introduction to the Study of Tropical Plankton.* Hutchinson Tropical Monograph, Hutchinson and Co., Ltd., New York, 160 pp.

WILLIAMS, A. B., 1965. Marine decapod crustaceans of the Carolinas. *U.S. Fish and Wildlife Ser., Fishery Bull.,* **65**: 1–298.

WILLIAMS, G., AND A. E. NEEDHAM, 1939. On relative growth in *Pinnotheres pisum. Proc. Zool. Soc. London,* **108A**: 539–556.

YEATER, L. W., 1965. Studies on the ecology of the commensal crab, *Pinnotheres maculatus* Say. *Masters thesis, The Florida State University,* Tallahassee, 60 pp.

Reference: *Biol. Bull.*, **145**: 492–508. (December, 1973)

RESPIRATORY VARIATION AND ACCLIMATION IN THE FRESHWATER LIMPET, *LAEVAPEX FUSCUS*[1]

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The North American "pond" limpet, *Laevapex fuscus* (C. B. Adams), is a member of the family Ancylidae; and the order Basommatophora (Basch, 1959, 1963 and Hubendick, 1964). It is one of the most highly evolved of the freshwater pulmonates in that the mantle-cavity is reduced and the main respiratory structure is a secondary gill formed from a double evagination of the left mantle-wall in the region of the rectum. General cutaneous exchange along with this "pseudobranch" allows *Laevapex* to be entirely aquatic in its respiration. It does not have to return to the surface periodically to renew air as in the majority of freshwater pulmonates. *Laevapex* is distributed throughout North America east of the Rocky Mountains in swamps, ponds, rivers, and lakes. It lives on rocks and other hard surfaces (Basch, 1959, 1963 and Hubendick, 1964).

The present work is concerned with the respiratory behavior of *Laevapex* and the correlation of this with both natural history and environmental variation. Earlier investigations involve the annual repiratory variation of three ancylid species: the European species, *Ancylus fluviatilis* and *Acroloxus lacustris* (Berg, 1951, 1952, 1953, and Berg, Lumbye and Okelman, 1958); and the North American stream limpet, *Ferrissia rivularis* (Burky, 1969, 1970, 1971). Like *Laevapex,* these ancylids all show reverse respiratory acclimation in winter-conditioned populations. The phenomenon of reverse acclimation appears to be paradoxically non-adaptive (Prosser, 1955). A major result of this study is a re-interpretation of the function of reverse acclimation in *Laevapex* within an appropriate ecological and environmental framework.

Specimens of *Laevapex fuscus* were collected bimonthly from three freshwater environments in upstate New York through the years 1970–1971. Concurrent studies of life-cycles, reproduction rates and bioenergetics (McMahon, 1972; see also McMahon, Hunter and Russell-Hunter, 1974) were made along with the assessments of respiration described in this report. Oxygen consumption rates were determined at both ambient and two standard temperatures for 100% oxygen tension on subsamples from each collection. For every 9 out of 10 of the seasonal samples, the effects of decreasing oxygen tensions at 20° C on rates of oxygen consumption were determined. In addition experiments were carried out on respiration rates before and after exposure to oxygen depleted water.

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METHODS

Three populations of *Laevapex fuscus* were investigated in this study. They occurred in Canandaigua Outlet (CAN) Canandaigua, New York (USGS map quadrangle, Canandaigua Lake, New York: 42°52′28″N, 77°16′08″W); Sterling Pond (FH) in Fair Haven, New York (USGS map quadrangle Fair Haven, New York: 43°20′30″N, 76°42′00″W); and the Erie Canal (ECF) just outside Fayetteville, New York (USGS map quadrangle, Syracuse East, New York: 43°02′50″N, 76°00′26″W). Large regular collections of *Laevapex fuscus* were taken at the CAN and FH sites from mid-August 1969 until late-December 1971. Erie Canal at Fayetteville was sampled regularly from June 1970 until late-November 1971.

Fair Haven and Erie Canal collections consisted entirely of specimens taken from rocks. Canandaigua Outlet samples came from rocks, bottles, metal cans and pieces of wood littering the bottom. At all three sites water temperatures varied between summer highs of 25–28° C and winter lows of 0–4° C (Fig. 1). During the warmer periods specimens of *Laevapex* were found on rock surfaces just within the oxidized surface layer of mud and organic debris. In the winter when water temperature is low, *Laevapex* migrates well down the rocks into very poorly oxygenated reducing mud and remains inactive until temperatures rise in the spring. Once ice forms in the winter oxygen tensions drop at all three sites due to diminished diffusion of oxygen from the surface (McMahon, 1972).

All respiration rates were monitored with Clark-type polarographic oxygen electrodes (Clark, 1956). The oxygen electrodes, respiration chambers and constant temperature apparatus was purchased from the Yellow Springs Instrument Company (Model-53). Respiration chambers were modified for snails according to the method of Burky (1969, 1971). Oxygen tension in the chambers was continuously recorded on a Honeywell Electronik-16 Strip Chart Recorder.

Specimens of *Laevapex fuscus* were returned to the laboratory in vacuum flasks and maintained at field temperature in an incubator. Respiration experiments were normally started within 24 hours of the collection. Rates were determined for one to four age-groups (premeasured as shell size-classes) intended as representative of the frequency distribution of the population sampled. The standard measurement for all samples was aperture length (AL), the greatest dimension anterior-posterior across the opening of the limpet shell. The class interval for each age group was ± 2.5 mm about a chosen aperature length within the range, one to eight millimeters (Snails were measured according to the method of Russell Hunter, 1953, 1961). Replicate experiments of the same size-classes were run occasionally as an indication of respiratory variance. After respiration measurements, experimental groups were taken to a constant dry weight in a 90° C oven (usully more than 12 hours). To assess shell biomass thirty or more snails were randomly selected from a summer sample at each site plus a winter sample at FH, and placed in 90° C pond water for one minute to separate each shell from its mantle tissue. For these separated shells, length and shell constant dry weight (90° C for three hours) were determined. Regressions were then computed for shell dry weights against aperture length (the longest dimension across the opening of the shell) for the ECF and CAN populations and both generations at FH.

Water used in the chambers was taken from the field at the time of collection, held at field temperature, and filtered twice before being used in any experiment.

Chamber water volume was always four milliliters. The oxygen probe was equilibrated at least twenty-five minutes in a blank chamber (*i.e.,* without limpets) before any experimental determinations were made.

The limpets were added to the experimental chamber once the water had stabilized at the experimental temperature and they remained nearly stationary after attachment for the course of the experiment. All sets of respiratory experiments consisted of runs at three temperatures (at field ambient temperature and at 10° C and 20° C). Although the first respiration run in each experimental sequence was done at field ambient temperature the temperature for the second run was chosen with reference to the recent environmental experience of the population.

Each experiment began at a saturation level of oxygen concentration assumed to be equal to that for distilled water at the experimental temperature and pressure. In the majority of runs, all rates were determined from the records of a continuously monitored change in oxygen concentration, either from 100% to 90%, if that 10% reduction was accomplished in less than 30 minutes, or from the reduction from 100% that actually occurs in 30 minutes.

Other experiments involved continuous monitoring at 20° C from 100% oxygen down to a tension at which respiration apparently stopped. For these more extensive runs, respiratory rates were normally calculated for every 10% change in concentration down to 30%, but, where rapid changes in rate occurred, the rates were determined at every five per cent decrease in concentration. In addition, another four series respiratory experiments were conducted. First, temperature acclimation was assessed in the laboratory by holding animals in an incubator at 20° C, after appropriate long runs, and then measuring their respiration every seven days until a major change in respiratory behavior could be detected. Secondly, respiratory change following long-term starvation was demonstrated by monitoring respiration up to three times every 24 hours at field ambient water temperature at the time of collection for a four to five day period following collection. Thirdly, response to low oxygen levels was followed by comparing normal respiratory behavior both to initial respiratory rates and to rates with decreasing oxygen tension of post-anaerobic snails. Finally the mortality due to sustained low oxygen stress was determined for each population (FH, CAN, ECF) by placing 20 snails in glass stoppered bottles containing deoxygenated field water maintained at field temperatures. Periodically, the number of snails still alive was recorded, and the time at which 50% of the snails died from low oxygen stress (LD-50) is presented below.

All respiratory rate values were computed as a rate per milligram "shell-free" dry tissue weight per hour (μl O_2/mg/hr). In this case, "shell-free" dry tissue weight equals the dry weight of the appropriate size-class minus the dry weight of its shells. Shell dry weights had been determined from a regression of dry weight against aperture length for each population.

RESULTS

Respiration experiments fall into three general classes: seasonal measurements of short-term respiration, temperature acclimation studies, and more specific investigations of low oxygen stress. Within each experimental set of size groups there was no consistent change in respiratory rate with change in mean dry tissue weight per animal. A similar lack of correlation between size and respiration rate has been reported for *Physa hawnii* (Daniels and Armitage, 1969). Therefore, oxygen uptake rates of all size groups in each experiment were averaged for each experimental temperature.

The experiments in which respiration rates were recorded over long periods of starvation showed that the variation in oxygen consumption rates resulting from starvation falls within the normal range of variation found in any single experiment. The consistent long-term effect of starvation on respiration rate reported for the European stream limpet *Ancylus fluviatilus* by Berg *et al.* (1958) does not occur in *Laevapex*.

Respiration rate in *Laevapex* is closely correlated to field water temperatures. During the winter when water temperatures drop to between 0°–2° C at all three sites, the respiration at ambient temperature and 100 per cent oxygen tension of overwintering specimens of *Laevapex* is correspondingly low (Fig. 1). For the months of January through March the average respiratory rate for CAN limpets was 0.223 $\mu l O_2$/mg/hr in 1970 and 0.161 $\mu l O_2$/mg/hr in 1971 (Fig. 1). The average winter rates for FH-L snails (FH-L is the overwintering generation of the bivoltine Sterling Pond population; see McMahon, 1972 and McMahon *et al.*, 1974) were 0.191 $\mu l O_2$/mg/hr in 1970 and 0.183 $\mu l O_2$/mg/hr in 1971 (Fig. 1). The ECF population had an average winter rate of 0.291 $\mu l O_2$/mg/hr during the months of March and November in 1971 (Fig. 1).

As temperature rises with the onset of spring, the respiration rate of the limpets shows a corresponding increase. Peak rates of respiration at 100 per cent oxygen tension and ambient temperature occur in July and August when field water temperatures are the greatest (25° C–30° C). At ECF, average summer respiration rates during July and August in 1971 were 2.010 $\mu l O_2$/mg/hr (Fig. 1). The CAN limpets had similar average rates of 1.818 $\mu l O_2$/mg/hr in 1970 and 1.759 $\mu l O_2$/mg/hr in 1971 (Fig. 1). The FH-E *Laevapex* population (FH-E is the spring generation of the bivoltine Sterling Pond population) had a very high average summer respiratory rate in 1970 of 7.312 $\mu l O_2$/mg/hr and a lower rate of 1.939 $\mu l O_2$/mg/hr in 1971 (Fig. 1). The lower summer rate in 1971 at FH may have resulted from the unusually low over night temperatures occurring that summer. Since Sterling Pond water temperature is normally within a few centigrade degrees of air temperature the 1971-E *Laevapex* population my have received a series of cold shocks which kept respiration rates at ambient temperature lower than usual.

Taken from each population in mid-summer, three groups of twenty specimens were held in pond water depleted of all oxygen at ambient temperature. The mean 50% mortality times (corresponding to LD-50's) for each set of sixty animals are: ECF, 27° C, LD-50 = 6 hours and 35 minutes; CAN, 23° C, LD-50 = 16 hours and 50 minutes; and FH, 24° C, LD-50 = 16 hours and 50 minutes. The overall mean LD-50 time for summer limpets in all three populations is 14 hours and 5 minutes. Similar values have been reported for other freshwater snails (Von Brand, Baernstein, and Mehlman, 1950).

After treatment with low oxygen stress in water depleted of all oxygen (zero oxygen tension) all limpets showed increased resipratory rates which were maintained for long periods. ECF limpets collected April 20, 1971 and held in water completely depleted of oxygen (zero O_2 tension) at 20° C for 48 hours showed

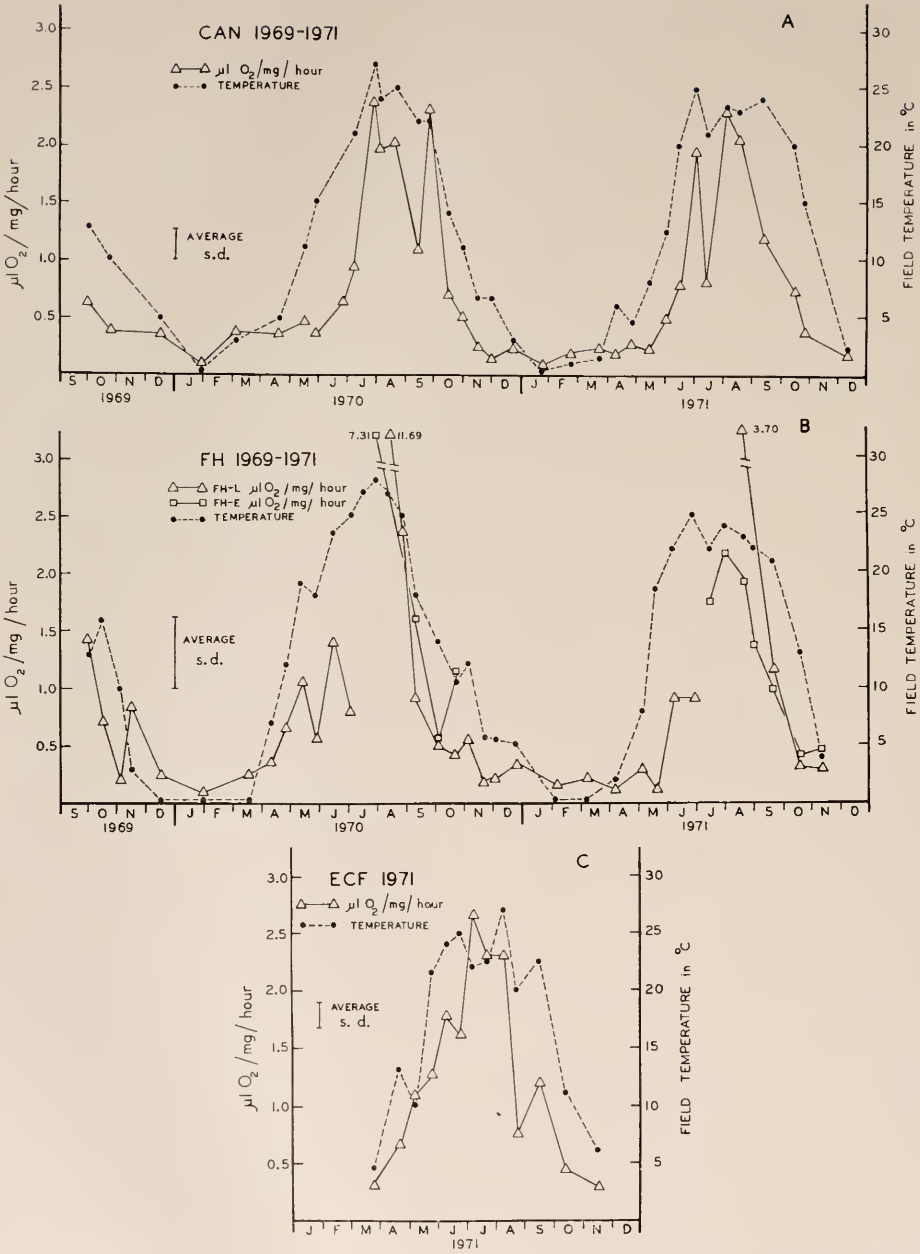

FIGURE 1. Respiration rates at ambient temperature. Oxygen consumption rates are in microliters oxygen per milligram shell-free dry tissue weight per hour at ambient field temperature. Open triangles, connected by solid lines, represent respiration rate and solid circles, connected by dotted lines, represent ambient field temperature (A, CAN; and B, FH, from late 1969 through 1971; C, ECF, in 1971). The points represent mean values of oxygen consumption rates for one to five separate groups of snails. The average s.d. indicates an average

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TABLE I

Mean midwinter and midsummer respiration rates at 10° and 20° C and 100 per cent oxygen saturation

		Winter January–March			Summer June–August		
		Average respiration rate in μlO_2 mg hr	Range	Q_{10}	Average respiration rate in μlO_2/mg hr	Range	Q_{10}
FH-L	20°C	0.554	0.48–0.62	2.01	1.338	0.69–2.56	1.48
	10°C	0.276	0.22–0.33		0.906	0.33–2.03	
CAN	20°C	0.553	0.48–0.69	2.38	1.277	0.33–2.24	1.26
	10°C	0.232	0.20–0.30		1.017	0.31–2.01	
ECF	20°C	0.917	0.84–0.99	2.28	1.503	0.74–2.20	1.76
	10°C	0.402	0.24–0.57		0.856	0.30–1.42	

no subsequent decrease in respiratory rate (from these increased rates) for 189 minutes after return to oxygen saturated water. A similar group of ECF limpets collected May 24, 1971, and exposed to oxygen depleted water (zero O_2 tension) for 37 hours showed no detectable short-term change in these increased rates after return to fully saturated water. Both groups of limpets showed just over a 100 per cent increase in respiratory rate immediately after treatment at all O_2 tensions when compared to normal rates before low oxygen stress (Fig. 2). The ECF limpets collected April 20, 1971 had respiration rates with decreasing oxygen tension remeasured 16 hours and 135 hours after return from low oxygen stress treatment to fully saturated water (Fig. 2A). This figure shows that the limpets appear to maintain their increased rates of respiration for more than 135 hours after they had been returned to water at 100 per cent saturation.

The ECF limpet group collected May 24, 1971 was expected for a second 43 hour period to oxygen depleted water (zero oxygen tension), following the first 37 hour low oxygen treatment and respiration measurement. Figure 28 shows that this second treatment increased respiration at all oxygen tensions to more than 4 times the pretreatment rates, and to twice that of the rates recorded after the first treatment.

Figure three shows the average respiratory rate of specimens of *Laevapex* at 100 per cent saturation at 10° C and 20° C throughout the years 1970 and 1971 for the three populations. During the winter months, when environmental water temperatures are between zero and five degrees centigrade, at 10° C and 20° C the limpets' respiration rates are very low. As water temperatures rise rapidly in June, the respiration rates also increase and they remain throughout the summer at more than twice the winter average (Fig. 3). Table I sets out the average winter and summer rates for all three *Laevapex* populations for the year 1971.

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of the standard deviations for all points which represent a mean of three or more separate determinations.

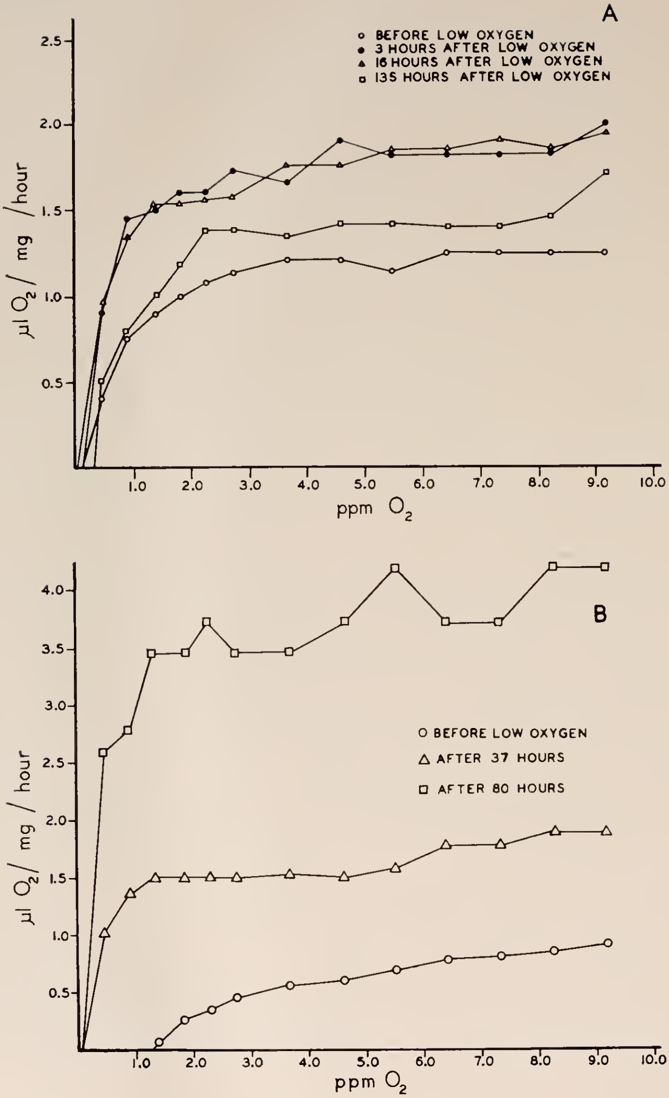

FIGURE 2. Respiration rate in microliters oxygen per milligram shell-free dry tissue weight per hour is plotted against oxygen tension in parts per million oxygen decreasing from right to left: (A) rate of oxygen consumption after low oxygen stress at 20° C. Limpets which have not been stressed with low oxygen conditions are shown as open circles. The solid circles represent respiration for the same limpets three hours after return to fully oxygen-saturated water from 48 hours of low oxygen stress; the triangles, 16 hours after return to fully saturated water from 48 hours of low oxygen stress; and the squares, 135 hours after return to fully saturated water from 48 hours of low oxygen stress; and (B) the effect of long-term low oxygen stress on oxygen consumption at 20° C. Limpets which have not been stressed with low oxygen conditions are indicated by circles. The triangles represent respiration rates for the same limpets after 37 hours in oxygen depleted water and the squares represent their respiration after 80 hours in oxygen depleted water. (Note that at 20° C and standard pressure, full saturation equals 9.17 ppm O_2.)

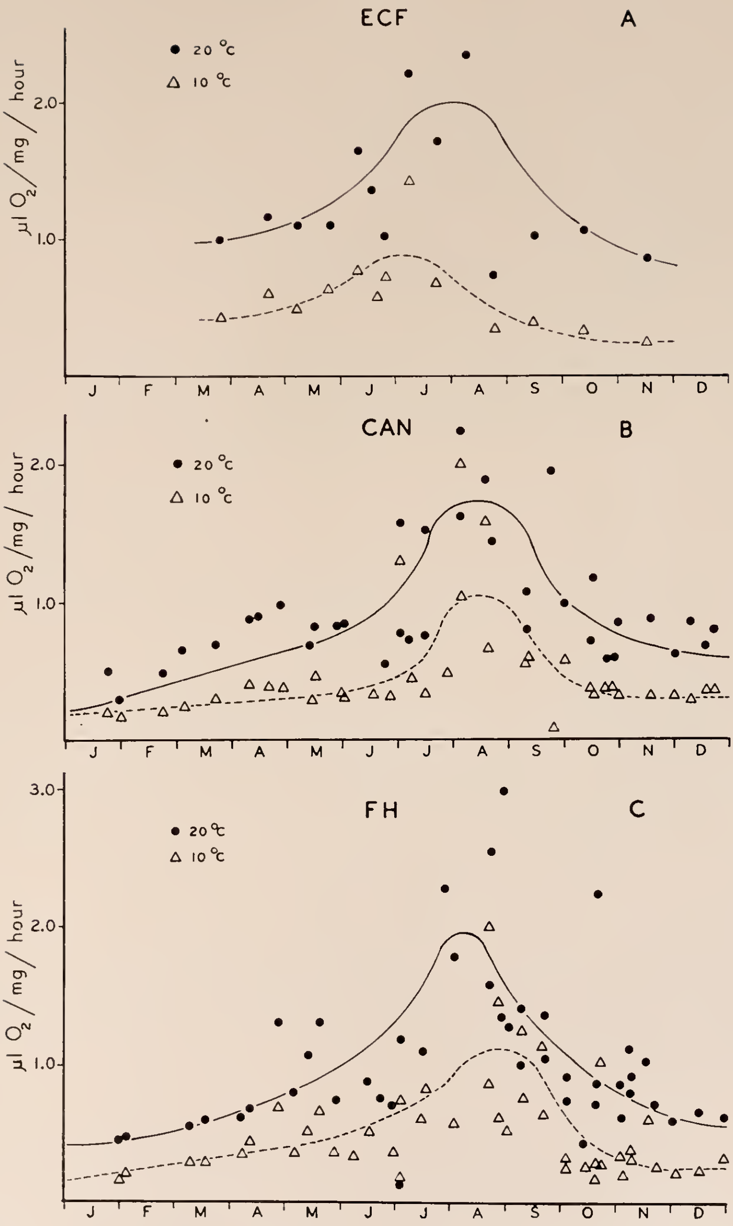

FIGURE 3. Rate of oxygen consumption at 10° C and 20° C throughout the year. Circles are oxygen consumption rate in microliters oxygen per milligram shell-free dry tissue weight per hour at 20° C and the triangles the rate at 10° C and at 100% oxygen tension. Acclimation is shown by the increase in rate during the summer months in the ECF (A), CAN (B) and FH (C) populations. Such a respiratory pattern has been previously called "reverse acclimation." For further explanation see text. The lines drawn on the three figures are purely arbitrary and assist only in the visualization of trends. The points represent mean values of oxygen consumption rates for one to five separate groups of snails.

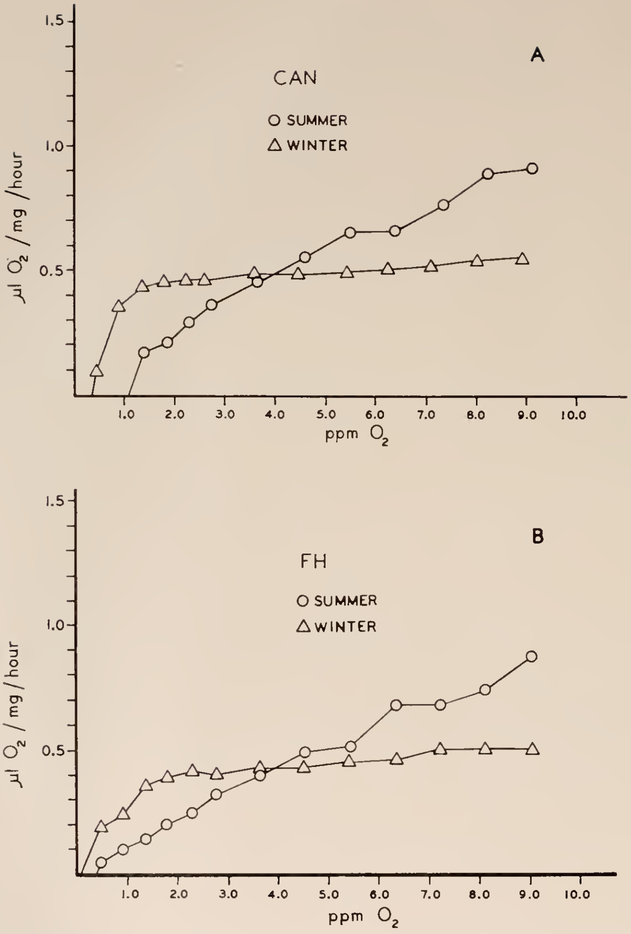

FIGURE 4. Respiratory acclimation. Oxygen consumption rate in microliters oxygen per milligram shell-free dry tissue weight per hour is plotted for decreasing oxygen tension at 20° C from right to left, for winter-conditioned (triangles) and summer-conditioned (circles) specimens of *Laevapex* from CAN (A) and FH (B). Note that at 20° C and standard pressure, full saturation equals 9.17 ppm O_2.

When respiratory rates are measured at any intermediate temperature, many poikilotherms which have been maintained at a lower temperature show a higher respiration rate than similar animals previously maintained at a higher temperature. This respiratory response to temperature conditioning over periods of about 7–20 days is called "Respiratory Acclimation." When the respiration rate of specimens of *Laevapex* from all three populations is followed at 10° C and 20° C at 100 per cent oxygen tension, limpets from a summer population accustomed to high ambient water temperatures have a greater respiratory rate than limpets from winter-conditioned populations accustomed to low ambient water temperatures when oxygen consumption is measured at the same temperatures (10° C and 20° C). This type of

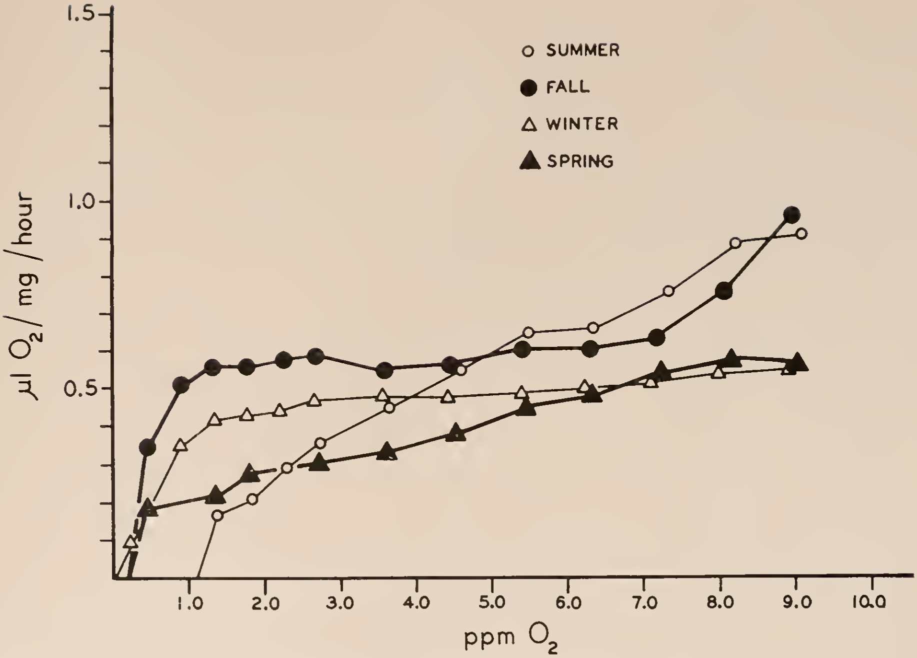

FIGURE 5. Respiratory acclimation with change in seasons. Respiration rate in microliters oxygen per milligram shell-free dry tissue weight per hour is shown with decreasing oxygen tension at 20° C from right to left, for limpets from the CAN population taken in midwinter (open triangles), spring (solid triangles), midsummer (open circles), and fall (solid circles). Note that at 20° C and standard pressure, full saturation equals 9.17 ppm O_2. The most obvious shifts from summer to fall and from winter to spring concern the left hand parts of the curves, that is, they are shifts in the limpets' responses to low oxygen tensions.

acclimation has previously been called "reverse acclimation." Similar respiratory acclimation has been reported for seven other invertebrates and one fish, and these instances will be listed and discussed below.

Long-run respiration experiments on *Laevapex* from these three populations show seasonal variation in respiratiory rate at low oxygen tensions. Summer-conditioned snails have higher initial respiratory rates at 100 per cent oxygen saturation at 20° C than (otherwise similar) winter-conditioned snails. However, summer-conditioned specimens of *Laevapex* are oxygen dependent in their respiration; as oxygen tension decreases in the respiration chamber, respiration rate also decreases (Fig. 4). Winter-conditioned limpets have lower initial oxygen uptake rates at 100 per cent oxygen saturation at 20° C, but these rates are maintained from 100 per cent to 10 per cent oxygen saturation and only after oxygen tension falls below 10 per cent is there any appreciable decrease in respiratory rates (oxygen independent respiration) (Fig. 4). Therefore, at lower oxygen tensions (below 40–50% O_2) specimens of *Laevapex* show a normal acclimatory response to temperature. The respiration rate of winter-conditioned animals is higher than that of summer-conditioned animals at these low tensions below 40–50% O_2 when mea-

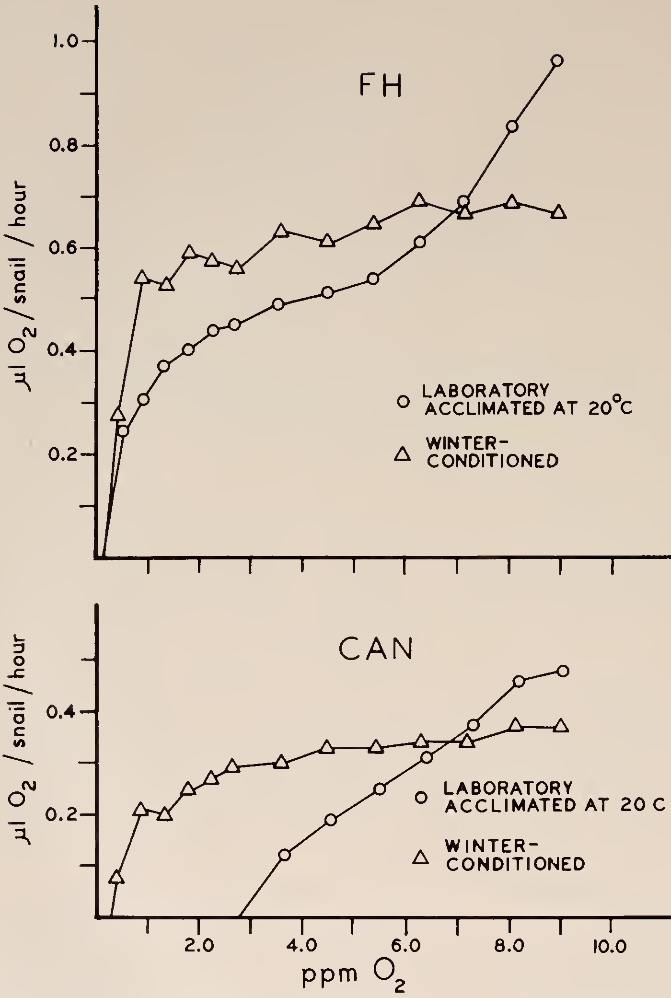

FIGURE 6. Respiratory acclimation in the laboratory. The vertical axes show respiration rate in microliters oxygen per snail per hour with decreasing oxygen tensions at 20° C from right to left, for winter-conditioned limpets from CAN and FH (triangles), and for the same limpets after being maintained in the laboratory at 20° C and 100% oxygen tension for 12 (CAN) and 20 (FH) days. Note that at 20° C and standard pressure, full saturation equals 9.17 ppm O_2.

sured at 20° C. Berg and Ockelmann (1959) investigated respiration rate with decreasing oxygen tension in nine freshwater snails. The respiration rates of the eight species were significantly diminished with decreasing oxygen tensions. Only *Bithynia leachi* showed an oxygen independent respiratory pattern similar to that of winter-conditioned *Laevapex*.

The snails shift from the summer to winter respiration pattern in the fall gradually as water temperature begins to drop and they gradually return to the summer respiration pattern in the late spring when water temperatures begin to rise. Figure 5 shows intermediate spring and fall respiration rate curves against oxygen tension at 20° C for the CAN population. These curves demonstrate the slow shifts which occur in these respiratory patterns.

Figure 6 shows respiration rate at 20° C plotted against oxygen tension for limpets collected from Canandaigua Outlet on March 21, 1971 (ambient water temperature 1.5° C) and from Fair Haven on April 6, 1971 (ambient water temperature 2.0° C). Both groups were kept at ambient temperature until the experiments were started. They both had typical winter curves with respiration rate decreasing only slightly as oxygen tensions decreased. After the initial long-run experiment the limpets were kept in an incubator at 20° C and at 100 per cent oxygen saturation (about 9.17 mgO_2/l). The respiration rate with decreasing oxygen tension was checked every three to four days thereafter until the limpets shifted to the summer respiratory pattern (Fig. 6). At 20° C and 100 per cent oxygen saturation, acclimation to the summer pattern took the CAN limpets 12 days and FH limpets 20 days to complete. The possible adaptational significance of these acclimation patterns will be discussed later.

Discussion

Annual variation of respiration rate at ambient temperatures have not been reported in detail for any mollusc populations. One Norwegian population of the oyster, *Ostrea edulis,* was shown to have a midsummer respiratory rate at 25° C 19 times greater than its mid-winter value at 0.0° C (Pederson, 1947). In two Danish populations, the European stream limpet, *Ancylus fluviatilis,* increases its repiratory rate during the summer temperature maxima by five to six times in one population (maximum at 13° C and minimum at 3° C) and in the other by 2.8 times (19° C and 7° C) their winter minimum rates (Berg, *et al.,* 1958). Burky (1969 and 1971) reported that maximum summer respiration of *Ferrissia rivularis* at 17° C was 10.5 times greater than the minimum winter value at 0.0° C. In the three populations of *Laevapex* of the present study FH-L had a 1971 maximum summer rate 36.0 times (0° C and 25° C) greater than the minimum winter rate (in 1970 this value was abnormally high at 127.1) (0° C and 28° C) while the value was 9.4 (4.5° C and 27° C) for ECF in 1971 and 26.6 (0° C and 26° C) and 26.2 (0° C and 25° C) in the CAN-70 and CAN-71 populations (Fig. 1). These differences in winter and summer respiration are considerably greater than those for *Ancylus fluviatilis* or *Ferrissia rivularis.* Since the change in respiration rate in specimens of *Laevapex* corresponds in part to change in ambient temperature, the large annual variation in ambient water temperature as recorded at the three sites may account for the large annual variation in respiration rates (Fig. 1). Except for the high respiration of the FH summer limpets in 1970 no significant differences were found between any of the three populations in respiration rate at corresponding ambient temperature.

Prosser (1955, 1967) and Bullock (1955) have reviewed acclimation in poikilothermic animals, and Segal (1961) has described some cases of acclimation in molluscs in great detail. Although the majority of poikilotherms including molluscs which have been investigated show normal acclimation (Bullock, 1955; Segal, 1961) reverse acclimation at 100% oxygen tension has been reported for natural overwintering populations in the goldfish (Roberts, 1960, 1966), in two species of barnacle (Barnes, Barnes and Finlayson, 1963) and in a freshwater gammarid (Krog, 1954). Among molluscan populations, reverse acclimation has been described for: a high intertidal limpet, *Patella vulgata* (Davies, 1965); a terrestrial pulmonate, *Helix pomatia* (Blazka, 1954); two European ancylid pulmonates, *Ancylus fluviatilis* and *Acroloxus lacustris* (Berg, 1951, 1952, 1953; and Berg *et al.,* 1958); and in the North American stream limpet *Ferrissia rivularis* (Burky, 1969, 1970, 1971). Two different interpretations of the adaptive significance of reverse acclimation to a particular species have appeared in the literature. The first hypothesis is that the reduction of respiration rate during the winter is an adaptation to the depletion of oxygen resulting from ice cover and subsequently reduced surface diffusion of oxygen (Roberts, 1960, 1966; Krog, 1954). These animals are said to be lowering their metabolic demand for oxygen as a response to its reduced availability in the environment. The other interpretation of the function of reverse acclimation is that in the winter when primary productivity is at a minimum and the animal is not actively feeding, then lower winter metabolic rates (reflected in decreased respiration rates) act to conserve energy stores during the overwintering period (Burky, 1969, 1971). Both of these interpretations involve paradoxes for the overwintering populations of *Laevapex* studied. *Laevapex,* like *Ferrissia* (Burky, 1971) shows an increase in total carbon and in C:N ratio just prior to overwintering (McMahon, 1972). As the majority of gastropods have been demonstrated to store carbohydrates (von Brand *et al.,* 1950; Goddard and Martin, 1966; Russell-Hunter, Meadows, Apley and Burky, 1968; and references within), this carbon increase in fall populations is assumed to be the result of a buildup of carbohydrate storage products before overwintering. But, just prior to winter, specimens of *Laevapex* crawl down rocks, into reducing mud where oxygen tensions are extremely low.

In poikilothermic animals which remain inactive during the winter, reverse acclimation to temperature is a paradoxical phenomenon (Prosser, 1955). To remain active at low temperatures, poikilotherms normally have to increase their metabolic rate over that of warm-acclimated animals of the same species (Prosser, 1955, 1967; Bullock, 1955).

Conservation of energy stores by reverse acclimation also presents a paradox. All previous reports of reverse acclimation, and my own results for initial respiratory rates, are based on respiration rates determined at 100% oxygen tension at the different experimental temperatures. In nature, *Laevapex* and other organisms can live in waters of very low oxygen tension. As is well known, anaerobic metabolism is highly inefficient and requires a much greater energy flow than does aerobic metabolism to provide an equal amount of energy output (von Brand *et al.,* 1950; Goddard and Martin, 1966, and references within). Under conditions of low oxygen tension in these inactive winter populations reverse acclimation of already low winter respiratory rates could make the animals partially anaerobic and highly inefficient in the metabolism of their energy stores. As is the case with *Laevapex,* in at least three previously reported cases of reverse acclimation: *Gammarus* (Krog, 1954); *Acroloxus laustris* (Berg, 1952); and goldfish (Roberts, 1960, 1966) the animals overwinter in oxygen depleted waters. Therefore, reverse respiratory acclimation as has been described at full oxygen saturation appears certainly no adaptation to temperature alone. Rather, reverse acclimation is merely a manifestation of a more general acclimatory change in the respiratory behavior of these animals from oxygen-dependent respiration at high temperatures to oxygen-independent respiration at low temperatures. This shift allows winter-conditioned animals to have a higher rate of respiration than summer-conditioned animals would have in such low oxygen winter environments. Such a respiratory shift is triggered by the decrease in water temperature in the fall and the shift conditions the animals to oxygen-independent respiration for their winter environment. Thus, temperature change is merely the signal for the acclimatory shift and is not the adaptationally significant factor. This new hypothesis is largely confirmed by the results of the respiration experiments with decreasing oxygen tension which show that in the summer when the limpets are active and in areas of high O_2 concentration they have higher initial respiratory rates at 100% oxygen saturation at 20° C than do winter-conditioned limpets. However, the respiration rate of summer-conditioned specimens of *Laevapex* is oxygen-dependent and decreases proportionately with decreasing oxygen tension (Fig. 4). Winter-conditioned limpets have lower initial oxygen uptake rates at full oxygen saturation at 20° C, but their respiration rate is independent of oxygen concentration and nearly the same oxygen uptake rate is maintained from 100% to 10% oxygen tension at 20° C. Only after oxygen tension falls below 10% (less than 1.0 mgO_2/l at 20° C) is there any appreciable rate decrease (Fig. 4). Therefore, given the lower oxygen tensions of reducing mud, the acclimatory processes of overwintering specimens of *Laevapex* could be described as positive rather than negative or reverse. The respiration rate of winter-conditioned animals is higher than that of summer-conditioned animals when both are measured at these low oxygen tensions (below 4.0 mgO_2/l) and at 20° C. This increase in respiratory rate at low oxygen tensions would seem to be an adaptation which allows *Laevapex* to metabolize aerobically and make efficient use of its carbohydrate energy stores during the period of winter inactivity.

Anaerobic metabolism in freshwater snails has been reviewed by von Brand (1946), and more recently by Goddard and Martin (1966). Most of the freshwater snails studied have been reported to repay an oxygen debt after experiencing periods of anaerobiosis (von Brand and Mehlman, 1953; Goddard and Martin, 1966; and references within). In contrast, the present work on *Laevapex* shows no overt repayment of an oxygen debt as it is normally described, even after long exposure to waters of very low oxygen concentration. Instead, exposure to oxygen depleted water in *Laevapex* seems to cause a general increase in respiratory rate at all O_2 tensions. This increased rate is maintained for at least 135 hours after return of the limpets to fully saturated conditions, and appears in part to be short-term acclimation of respiratory rate to low O_2 tensions. Prosser (1955) points out that some acclimatory shifts are accomplished in only 17–24 hours. In 18 species of freshwater snails von Brand, *et al.*, (1950) found that anaerobic metabolism resulted in temporally increased levels of lactic acid. But, while lactic acid was retained in the tissues of all physids and lymnaeids tested, it was the major end-product of anaerobiosis in only two species. In contrast, in the planorbids and operculates tested, lactic acid was continuously excreted and there was no build-up (von Brand *et al.*, 1950). Specimens of *Laevapex* increase their total carbon content and C:N ratio prior to overwintering (McMahon, 1972). Such stores are probably carbohydrates (Russell-Hunter *et al.,* 1968; Goddard and Martin, 1966; and references within) and may be utilized to survive periods of extremely low oxygen tension encountered by overwintering snails. Such snails migrate into reducing mud. In this respect *Laevapex* is much like the goldfish which also shows reverse acclimation (Roberts, 1960, 1966). Specimens of the crucian carp taken from winter habitats of low oxygen tension were shown to pay no normal oxygen debt after long periods of anaerobiosis. Instead of lactic acid, fat and higher fatty acids appeared to be the end-products of an anaerobic metabolism (Blazka, 1958). Similarly, of 11 freshwater snails, those with low resistance to anaerobiosis were shown to accumulate lactic acid in the blood during low oxygen stress, while those more resistant to low oxygen stress appear to accumulate less toxic higher fatty acids (Mehlman and von Brand, 1951). Freshwater snails under anaerobic stress will excrete lactic acids and the other end-products of metabolism (von Brand *et al.*, 1950; von Brand and Mehlman, 1953). Because of the small tissue volume of *Laevapex* most possible end-products of anaerobic metabolism could be readily diffused to the environment or actively excreted. Without metabolite accumulation during anaterobiosis, there need be no overt payment of an oxygen debt on return to oxygen saturated water.

I wish to express my gratitude to Dr. W. D. Russell-Hunter for his assistance and guidance in all phases of this work.

Summary

1. From three populations of *Laevapex fuscus* samples were collected bi-weekly through 1970 to 1972. Oxygen consumption rates at ambient temperature and at 10° C and at 20° C were determined. Other respiration experiments involved decreasing O_2 tension at 20° C and the consequences of low oxygen stress.

2. At ambient temperatures, respiration during the summer is normally 26 to 36 times the winter respiratory rate.

3. After treatment with low oxygen stress there is no overt payment of an oxygen debt in *Laevapex,* instead there is a general increase in respiratory rate at all oxygen tensions. These rates are maintained for up to 135 hours after return to fully saturated conditions and appear to be in part a short-term acclimation of respiratory rates to low oxygen tensions.

4. Measured at 100% oxygen tension at 10° and 20° C, respiration rates are higher in the summer than in the winter for all three populations. Such a respiratory response to temperature has been called reverse acclimation. *Laevapex* lives in conditions of low oxygen tension during the winter. The respiration experiments with decreasing oxygen tension show that at low oxygen tensions winter-conditioned specimens have a *higher* respiratory rate than summer-conditioned individuals. Such a response to low oxygen tensions may be interpreted as a positive rather than reverse acclimation.

5. A hypothesis is advanced regarding "reverse" acclimation in this species. Reverse acclimation appears to be merely one manifestation of a more general accli-

matory change in winter-conditioned *Laevapex*. These limpets, when winter-conditioned, have a higher respiratory rate at low oxygen tension than do summer-conditioned limpets. Although this acclimatory process appears to be triggered by decreasing temperature, its adaptational significance involves the survival of the inactive overwintering limpet in contact with reducing mud.

LITERATURE CITED

Barnes, H., M. Barnes and D. M. Finlayson, 1963. The seasonal changes in body weight, biochemical composition, and oxygen uptake of two common arctic cirripedes, *Balanus balanoides* and *B. balanus*. *J. Mar. Biol. Ass. U. K.*, **43**: 185–211.

Basch, P. F., 1959. The anatomy of *Laevapex fuscus*, a freshwater limpet (Gastropoda: Pulmonata). *Misc. Publ. Mus. Zool. Univ. Mich.*, **108**: 1–56.

Basch, P. F., 1963. A review of the recent freshwater limpet snails of North America (Mollusc: Pulmonata). *Bull. Mus. Comp. Zool. Harvard Univ.*, **129**: 401–461.

Berg, K., 1951. On the respiration of some molluscs from running and stagnant water. *Annee Biol.*, **55**: 561–563.

Berg, K., 1952. On the oxygen consumption of Ancylidae (Gastropoda) from an ecological point of view. *Hydrobiologia*, **5**: 225–267.

Berg, K., 1953. The problem of respiratory acclimation. *Hydrobiologia*, **6**: 331–350.

Berg, K., J. Lumbye and K. W. Ockelmann, 1958. Seasonal and experimental variations of the oxygen consumption of the limpet *Ancylus fluviatilis*. *J. Exp. Biol.*, **35**: 43–73.

Berg, K., and K. W. Ockelmann, 1959. The respiration of freshwater snails. *J. Exp. Biol.*, **36**: 690–708.

Blazka, P., 1954. Temperaturadaptation des gesamtmetabolis mus bei der weinbergschnecke *Helix pomatia* L. *Zool. Jahr. Abt. Allg. Zool. Physiol.*, **65**: 130–138.

Blazka, P., 1958. The anaerobic metabolism of fish. *Physiol. Zoöl.*, **31**: 117–138.

Bullock, T. H., 1955. Compensation for temperature and activity of poikilotherms. *Biol. Rev.*, **30**: 311–342.

Burky, A. J., 1969. Biomass turnover, energy balance, and interpopulation variation in the stream limpet, *Ferrissia rivularis* (Say) with special reference to respiration, growth, and fecundity. *Ph.D. dissertation, Syracuse University*, 105 pp (*Diss. Abstr.*, **30**: 3917-B, order number 70–1942).

Burky, A. J., 1970. Reverse acclimation at low temperature in the stream limpet, *Ferrissia rivularis* (Say). *Acta Cient. Venez.*, **21**: 28.

Burky, A. J., 1971. Biomass turnover, respiration, and interpopulation variation in the stream limpet, *Ferrissia rivalaris* (Say). *Ecol. Monogr.*, **41**: 235–251.

Clark, L. C., Jr., 1956. Monitor and control of blood and tissue oxygen tensions. *Trans. Amer. Soc. Artif. Internal Organs*, **2**: 41–57.

Daniels, J. M., and K. B. Armitage, 1969. Temperature acclimation and oxygen consumption in *Physa hawnii* Lea (Gastropoda: Pulmonata). *Hydrobiologia*, **33**: 1–13.

Davies, P. S., 1965. Environmental acclimation in the limpet, *Patella vulgata* L. *Nature*, **205**: 924.

Goddard, C. K., and A. W. Martin, 1966. Carbohydrate metabolism. Pages 275–308 in K. M. Wilbur and C. M. Yonge, Eds., *Physiology of Mollusca*, Vol. II. Academic Press, New York and London.

Hubendick, B., 1964. Studies on Ancylidae, the subgroups. *Goteborgs Kungl. Vetenskaps-Och Vitterhets-Samhalles Handlingar.*, **B9**(6): 1–72.

Krog, H., 1954. Temperature sensitivity of freshwater gammarids. *Biol. Bull.*, **107**: 397–410.

McMahon, R. F., 1972. Interpopulation variation and respiratory acclimation in the bioenergetics of *Laevapex fuscus*. *Ph.D. dissertation, Syracuse University*, 164 pp. (*Diss. Abstr.*, **33**: 5068-B, order number 73–9549).

McMahon, R. F., R. D. Hunter and W. D. Russell-Hunter, 1974. Variation in *Aufwuchs* at six freshwater habitats in terms of carbon biomass and of carbon:nitrogen ratio. *Hydrobiologia*, in press.

Mehlman, B., and T. von Brand, 1951. Further studies on the anaerobic metabolism of some freshwater snails. *Biol. Bull.*, **100**: 199–205.

PEDERSON, E., 1947. Østersens respirajon. Under-søkelser utørt ved statens Utklekningsanstalt Flødevigen. *Fiskeridir. Ski. Havenderøk.*, **8**: 1–51.

PROSSER, C. L., 1955. Physiological variation in animals. *Biol. Rev.*, **30**: 229–262.

PROSSER, C. L., 1967. *Molecular Mechanisms of Temperature Adaptation.* Symposium American Association Advancement of Science, Washington, D.C., 398 pp.

ROBERTS, J. L., 1960. The influence of photoperiod on thermal acclimation. *Zool. Anz.*, **24**: 73–78.

ROBERTS, J. L., 1966. Systematic versus cellular acclimation to temperature by poikilotherms. *Helgolaender Wiss. Meeresunters.*, **14**: 451–465.

RUSSELL HUNTER, W. D., 1953. On the growth of the fresh-water limpet, *Ancylus fluviatilis* Muller. *Proc. Zool. Soc. London*, **123**: 623–636.

RUSSELL HUNTER, W. D., 1961. Annual variations in growth and density in natural populations of freshwater snails in the west of Scotland. *Proc. Zool. Soc. London*, **136**: 219–253.

RUSSELL-HUNTER, W. D., R. T. MEADOWS, M. L. APLEY AND A. J. BURKY, 1968. On the use of a "wet oxidation" method for estimates of total organic carbon in mollusc growth studies. *Proc. Malacol. Soc. London*, **38**: 1–11.

SEGAL, E., 1961. Acclimation in molluscs. *Amer. Zool.*, **1**: 235–244.

VON BRAND, T., 1946. Anaerobiosis in invertebrates. *Biodynamica Monographs*, **4**: 1–328.

VON BRAND, T., H. D. BAERNSTEIN AND B. MEHLMAN, 1950. Studies on the anaerobic metabolism and the aerobic carbohydrate consumption of some freshwater snails. *Biol. Bull.*, **98**: 266–276.

VON BRAND, T., AND B. MEHLMAN, 1953. Relations between pre- and post anerobic oxygen consumption and oxygen tension in some freshwater snails. *Biol. Bull.*, **104**: 301–312.

Reference: *Biol. Bull.*, **145**: 509–531. (December, 1973)

THE BIOLOGY AND FUNCTIONAL MORPHOLOGY OF *LATERNULA TRUNCATA* (LAMARCK 1818) (BIVALVIA: ANOMALODESMATA: PANDORACEA)

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The subdivision of the class Bivalvia has been and still is, a source of controversy. Most systematists agree that the Protobranchia are distinct from the Lamellibranchia (Ridewood, 1903; Pelseneer, 1911; Owen, 1959; Purchon, 1959; Yonge, 1959; J. E. Morton, 1967). On the other hand the scavenging Septibranchia (Yonge, 1928) are considered by some authorities, *e.g.*, Purchon (1962) to be derivable directly from a protobranch ancestor. Alternatively some early workers (Ridewood, 1903; Douvillé, 1912) and the majority of present day authorities, *e.g.*, Newell (1965); J. E. Morton (1967) and Taylor, Kennedy and Hall (1972) regard the septibranchs as being highly specialized members of the Lamellibranchia. In such classification schemes they have usually been ordered with a group of highly specialized lamellibranchs, the so-called Anatinacea, in the sub-order (Thiele, 1935) or sub-class (Newell, 1965) Anomalodesmata. J. E. Morton (1967) puts the Septibranchia (Poromyacea) in the same class as, but in a different order from, the remaining "Anatinacea," the Pandoracea and Clavagellacea.

Most modern authorities agree on the classification scheme proposed by Newell (1965), *e.g.*, Taylor, *et al.* (1972) in which the sub-class Anomalodesmata is divided into four Super-families, the rare Pholadomyacea, the Pandoracea (including *Laternula*), the Poromyacea (the septibranchs) and the Clavagellacea. This scheme of classification has been adopted in this paper.

The Poromyacea are not well known, the most adequate account of two of them, *i.e.*, *Cuspidaria* and *Poromya* being by Yonge (1928). Little is known about the Pholadomyacea (Runnegar, 1972) and only *Brechites penis* in the Clavagellacea having been studied in any great detail by Purchon (1956, 1960), although Soliman (1971) has recently reported upon a species of *Clavagella*.

The Pandoracea are the best known, such genera as *Pandora* and *Cochlodesma* having been studied by Allen (1945, 1958) and *Entodesma* and *Mytilimeria* by Yonge (1952). In relative terms, however, this assemblage too is poorly known. For this reason a detailed study has been undertaken of *Laternula truncata* (Lamarck 1818).

MATERIALS AND METHODS

Specimens of *Laternula truncata* were collected from various sites in Hong Kong at irregular intervals. One of the better collecting sites was Tai Po marshes in Tolo Harbour.

Specimens for routine serial sectioning were fixed in alcoholic Bouin Duboscq and stained in either Heidenhain's haematoxylin, Masson's trichrome or Mallory's triple stain.

Portions of the ctenidial filaments and the style sac, were treated in the same way and sectioned transversely.

Portions of the digestive diverticula and of the kidney were also fixed in Zenker's fixative and subsequently stained in Heidenhain's hematoxylin.

Five optic tentacles were fixed in Zenker's fixative and stained in Heidenhain's hematoxylin while a further five were fixed in 5% formal saline and stained for nervous tissue by Holme's silver method (Carleton and Drury, 1957). Half of the eyes were sectioned transversely, the remainder longitudinally.

The ciliary currents elucidated in this work were demonstrated by the application of suspended carmine particles in sea water.

Results

Ecology

The Anomalodesmata comprise a diverse assemblage of bivalves which are generally adapted to infaunal life. They occur sublittorally, *e.g., Thracia* (Yonge, 1937), at low tide on muddy shores, *e.g., Pandora* (Allen, 1954), *Brechites* (Purchon, 1956, 1960) and under stones on the shore, *e.g., Entodesma* (Yonge, 1952). *Mytilimeria* is adapted to a highly specialized mode of life embedded in the tests of ascidians (Yonge, 1952). *Lyonsia* (Ansell, 1967), *Mytilimeria* and *Entodesma* (Yonge, 1952) possess a byssus. *Cuspidaria* and *Poromya* are members of the infanual benthos (Yonge, 1928). Some clavagellids are found in soft rocks and corals (Soliman, 1971).

In the Periplomatidae, *Cochlodesma* (Allen, 1958) occurs in fine gravels from extreme low water mark to a depth of 60 fathoms. Vohra (1971) has recently shown that *Laternula anatina* occurs on sandy shores at all levels except the uppermost ones. I have found *Laternula tasmanica* over a wide range of tidal levels on the sandy shores of New South Wales, Australia although the animal is particularly prevalent in estuarine sands and is most commonly found burrowing in *Zostera* beds. Purchon (1958) reported *Laternula rostrata* (=*Laternula truncata*) as occurring on the seaward side of mangrove formations in Singapore. This is also the habitat occupied by this species in Hong Kong, although it has only been found on shores of coarse sand, *e.g.,* Tai Po marshes in Tolo Harbour. In Deep Bay, where there is a muddy shore on the seaward side of the mangroves, *L. truncata* is only found in localized patches of coarse sand or gravel. As far as is known from the available records *L. truncata* is distributed from Japan in the North (Kira, 1962) to Singapore in the South (Purchon, 1958).

Unlike *Thracia* (Yonge, 1937) and *Cochlodesma* (Allen, 1958), the species of *Laternula* upon which we have information do not build true burrows. They are always found lying vertically in the sand with the siphons pointing upwards. *Cochlodesma* lies horizontally (Allen, 1958). A large specimen of *L. truncata, i.e.,* of shell length of 6 cm, would be found at a depth of 9 cm, the siphons extending to the surface. When dug up, the animal showed no escape reactions, indeed in nearly all cases the siphons were not withdrawn. Once disturbed, the adult animal is incapable of reburrowing; the foot being small and of no use in this respect. This observation was subsequently verified in the laboratory. *L. truncata* can be regarded as a "passive burrower" as *Pholadomya* is assumed to be (Runnegar, 1972). Siphonal retraction is always very slow.

Young specimens of *L. truncata* (and *L. tasmanica*) possess a relatively large, mobile foot and the animal can burrow when disturbed.

Functional morphology

The shell and ligament. The shell of *Laternula truncata* is inequivalve, the left valve being somewhat deeper than the right and overlapping it slightly. The shell valves are markedly inequilateral. The anterior region of the shell is enlarged, whilst the posterior region is narrower and elongated, housing the long siphons when they contract (Fig. 1). There is a deep and broad pallial sinus (PS) to the pallial line (PL). The pallial line is wide.

The shell is pearly white in color both internally and externally, particularly in the dorsal region of the shell where much of the calcareous material has been eroded away perhaps due to the acid mangal soil (Macnae, 1968). Marginally a wide band of thinly distributed calcareous material is present. Taylor, *et al.* (1972) have shown that all of the Pandoracea (except the Thracidae) have a characteristic shell microstructure of inner and middle nacreous layers and a thinly distributed outer prismatic layer. Conditions in *L. truncata* are similar.

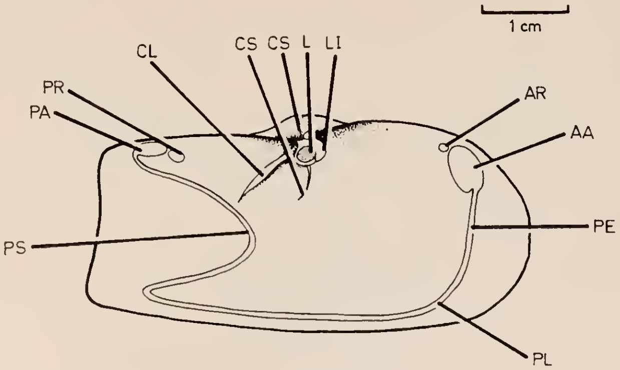

FIGURE 1. *Laternula truncata,* interior view of the left shell valve. Abbreviations are: AA, Anterior adductor muscle scar; AR, Anterior pedal retractor muscle scar; CL, Clavicle; CS, Crack in shell; L, Ligament; LI, Lithodesma; PA, Posterior adductor muscle scar; PE, Pedal gape; PL, Pallial line; PR, Posterior pedal retractor muscle scar; PS, Pallial sinus.

The periostracal layer is brown in color and rough in texture. Marginally it is thick, although over the rest of the shell it is thin. Dorsally, on either side of the primary ligament the periostracum of the left and right sides fuses uniting the shell valves in these regions (Fig. 2, FP). A similar situation has been reported for *Pandora* and *Cochlodesma* (Allen 1954; 1958) and *Entodesma* and *Mytilimeria* (Yonge, 1952).

When closed the shell valves only meet mid-ventrally and mid-dorsally at the ligament. There is a small anterior pedal gape (PE) and an extensive posterior siphonal gape.

The overall dimensions of the shell of *L. truncata* are comparatively regular as can be seen from the estimate of the ratio of width:height:length which is

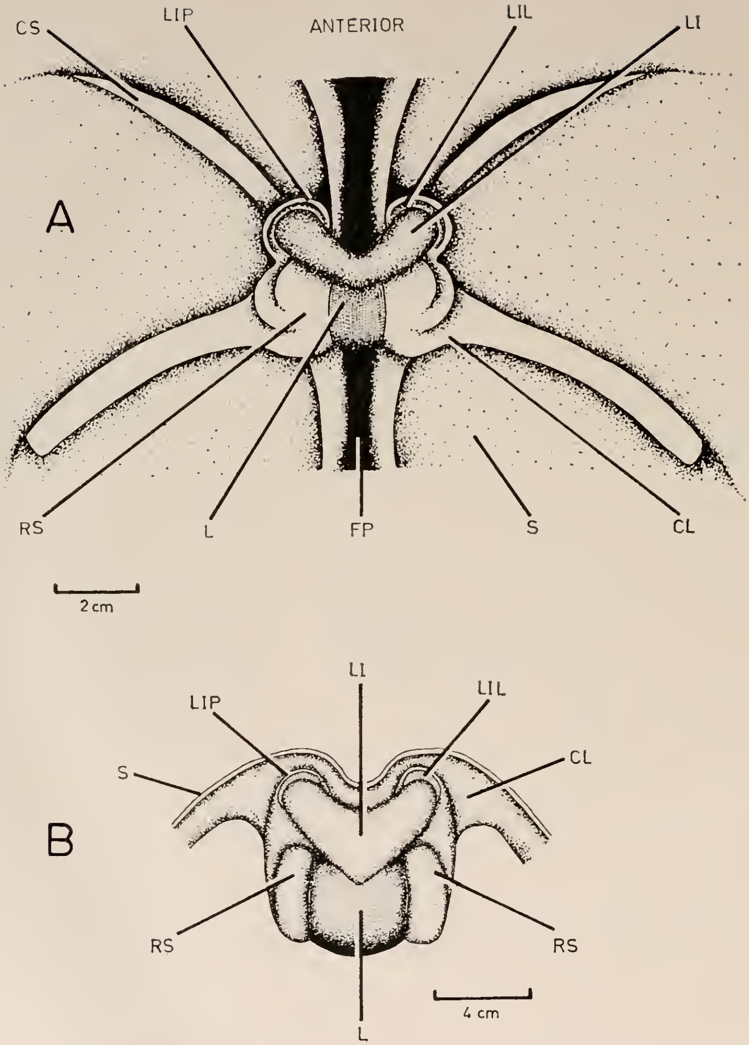

FIGURE 2. *Laternula truncata,* the ligament and lithodesma viewed from (A) the ventral and (B) from the anterior aspect. Abbreviations are: CL, Clavicle; CS, Crack in shell; FP, Fused periostracum; L, Ligament; LI, Lithodesma; LIL, Lithodesmal ligament; LIP, Lithodesmal plate; RS, Resilifer; S, Shell.

1:1.31 ± 0.19:3.18 ± 0.56. These figures suggest that shell length is one of the most variable factors in the growth of the shell. The shell is thin. The largest specimen of *L. truncata* examined had dimensions of: width 1.90 cm, height 2.60 cm, length 6.0 cm, and a shell thickness of, mid anteriorly, only 0.2 mm. This is due to the reduction of the prismatic layer of the shell.

A crack in each shell valve (Figs. 1 and 2, CS) extends ventrally from the umbo to approximately half the height of the shell. The anterior edge of the crack overlies the posterior edge, thereby preventing entry of terrigenous material, but at the same time allowing the bending of the dorsal border of the shell, when the adductor muscles contract.

The ligament (Fig. 2, L) of *L. truncata* is small and is located on a resilifer (RS) from which also radiate laterally the posterior major shell supporting struts or clavicles (CL) which serve to strengthen the thin shell. Anterior to, and fused to, the ligament is a boomerang shaped calcareous ossicle, or lithodesma (LI), which is also attached by ligamental pads (LIL) to the lithodesmal plates (LIP) of the resilifer. The lithodesma effectively prevents expansion of the ligament when the adductor muscles relax. A lithodesma, though of different shapes, is also found in *Myodora*, *Myochama* and *Chamostrea* (Thiele, 1935) and in *Mytilimeria* and *Entodesma* (Yonge, 1952). The ligament of the periplomatid *Cochlodesma praetenue* has been described by Allen (1958, 1960) as being unusual in form but basically opisthodetic. Surprisingly Allen did not notice that a large lithodesma is present in this species. The ligament of *L. truncata* is very similar to that of *Cochlodesma*.

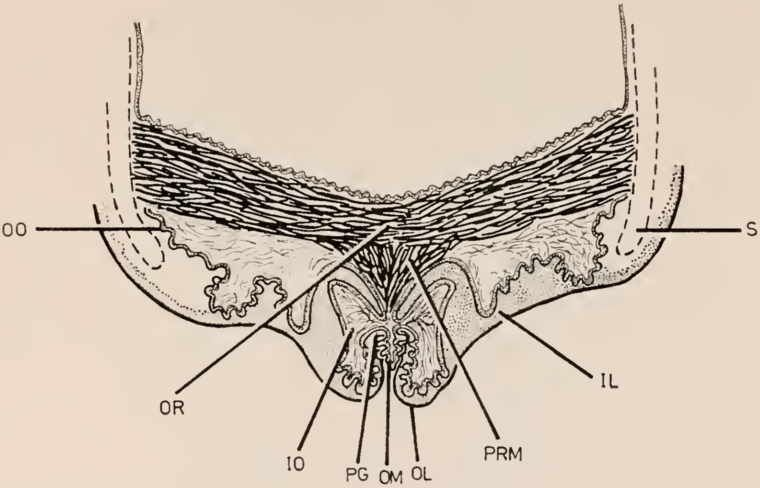

FIGURE 3. *Laternula truncata*, a transverse section through the ventral mantle margin. Abbreviations are: IL, Inner periostracal layer; IO, Inner layer of outer mantle fold; OL, Outer periostracal layer; OM, Fused outer layers of middle mantle fold; OO, Outer layer of outer mantle fold; OR, Orbital muscle; PG, Periostracal groove; PRM, Pallial retractor muscle; S, Shell.

The mantle. Fusion of the marginal folds of the mantle occurs dorsally above the exhalant siphon and also anterior to the pedal gape. In these regions the fusion is by way of the inner and middle folds and the inner surfaces of the outer folds and is thus of type C (Yonge, 1957). Such an arrangement accounts for the fusion of the periostracum already alluded to in this region and which forms a secondary "external" ligament. Such a situation was originally regarded by Owen Trueman and Yonge (1953) as primitive, but subsequently Yonge (1957), at least, changed his mind, and considered this feature to be secondary.

Ventrally, that is between the inhalant siphon and the pedal gape, mantle fusion involves only the inner folds and the inner surfaces of the middle mantle folds and is thus of type B (Yonge, 1957) (Fig. 3). Such a condition is not, apparently, characteristic of the Anatinacea studied earlier by Yonge (1952, 1957) in which all mantle fusion is apparently of type C. In *Laternula truncata* anterior and posterior to the mid point of the ventral border the fusion gradually involves the outer surfaces of the middle mantle folds and the inner surfaces of the outer mantle folds to give the type C condition, described above.

There is a small pedal aperture located antero-ventrally, but there is no fourth pallial aperture in *L. truncata* as there is in *Entodesma* and *Mytilimeria* (Yonge, 1952).

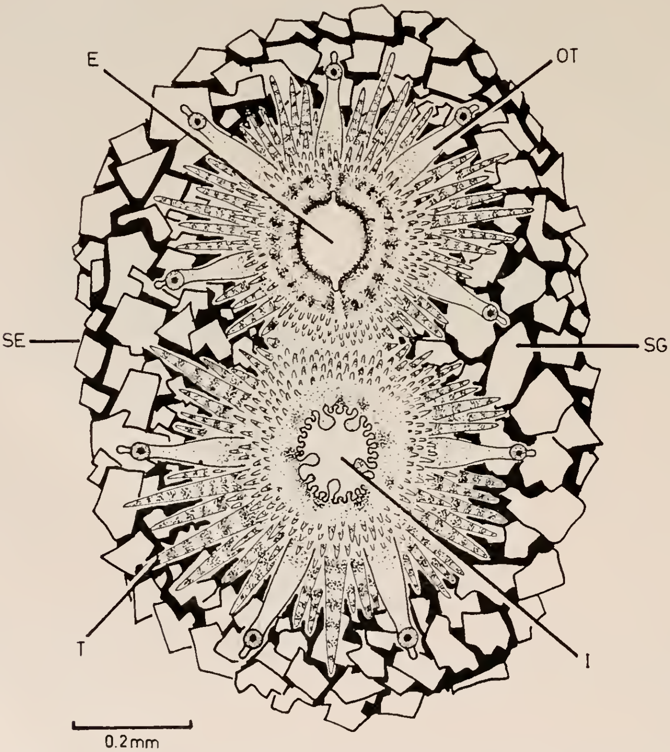

FIGURE 4. *Laternula truncata*, a posterior view of the siphons with the animal actively filtering fresh sea water. Abbreviations are: E, Exhalant siphon; I, Inhalant siphon; OT, Optic tentacle; SE, Siphonal edge; SG, Sand grains; T, tentacle.

The periostracum is composed of two layers. The outer layer (OL) is thin (2.5–5.0 μ) and stains bright red with Masson's trichrome and Mallory's triple stain. The inner layer (IL) stains bright blue with both of these stains and as noted earlier is very thick marginally (50–100 μ). In other regions of the shell, however, this layer is thin (10 μ). It is secreted by the inner surface of the outer mantle fold (IO). A similar two layered periostracum is also found in *Dreissena polymorpha* (B. S. Morton, 1969) and *Galeomma takii* (B. S. Morton, 1973b) though the situation is more complicated in various mytilid genera (Beedham, 1958; B. S. Morton, 1973a) where the periostracum is composed of three layers.

The mantle is exceptionally thin over the general surface, except ventrally where it contains muscles which unite the valves and which can be referred to as orbital muscles (OR). Pallial retractors (PRM) serve to withdraw the mantle margins into the shell, presumably when the animal is disturbed. The occurrence of such large muscles uniting the two valves accounts for the wide pallial line and suggests that these muscles may be important in movement of the shell valves. Orbital muscles are also well developed in the Saxicavacea (Russell Hunter, 1949; Yonge, 1971).

The siphons. The combined siphons of *Laternula truncata* when fully extended are almost half as long as the shell nd are formed by fusion of all three folds of the mantle margin including the periostracal groove and are thus of type C (Yonge, 1957). The thick periostracum that surrounds them is covered by sand grains (Fig. 4, SG) which, as suggested by Yonge (1952) for *Entodesma saxicola,* indicates that the periostracum was sticky when secreted and subsequently hardened after the sand grains had adhered. The siphons are capable of total, though slow, retraction. The extensive posterior emargination of the shell valves prevents them from enclosing the siphons. As noted earlier the pallial sinus is deep and broad. The tips of both inhalant (I) and exhalant (E) siphons bear a crown of (a) tactile tentacles (Fig. 4, T) and also possess eyes mounted on special (b) optic tentacles (OT). The exhalant siphon possesses five such eyes whilst the inhalant siphon possesses four. These numbers were constant for all specimens examined. No regular number or order in the arrangement of the tactile tentacles could be recognized, but as a general rule the larger tentacles occupied an outer position. The exhalant aperture closes by contraction at two lines of folding, while the inhalant siphon closes by contraction at five lines of folding. The tentacles are delicately patterned by brown, yellow and white bands, which may be a form of disruptive

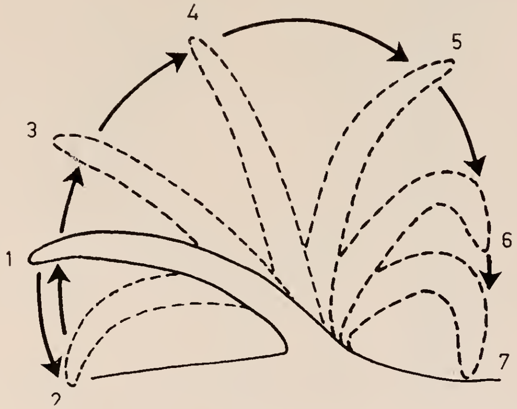

FIGURE 5. *Laternula truncata,* sequential stages in the flicking action of a single siphonal tactile tentacle.

coloration, the siphons being difficult to discern against a background of sand grains. The siphons themselves are also camouflaged by the covering of sand grains.

The tactile tentacles of the siphons and to a lesser extent the optic tentacles undergo, at certain times, a curious flicking motion. The resting tentacle (Fig. 5, 1) bends initially outwards (2) and then rapidly flicks inwards towards the siphonal aperture (2–7), flexing as it does so. This process is repeated regularly. It has been established that they perform these flicking motions: (a) just before the siphonal apertures open; (b) immediately after the siphonal apertures closed subsequent to the animal being disturbed; and (c) when the light falling upon the siphons was temporarily obstructed, *i.e.*, a "shadow" reflex; the siphonal apertures also close.

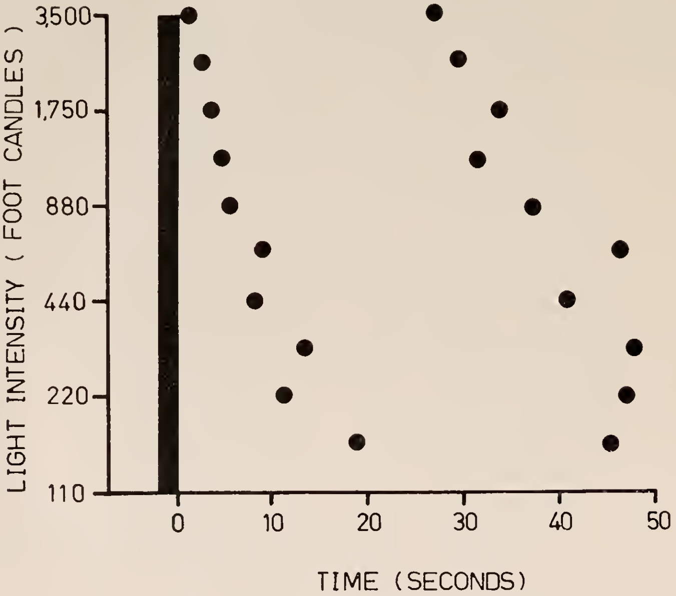

FIGURE 6. *Laternula truncata*, a graph showing the time of onset and the time of cessation at different light intensities, of flicking of the siphonal tentacles after a two second dark phase (the black column). Each point represents the mean of six separate observations.

In the latter case a lag period was observed between the onset of the dark phase and the onset of beating by the tentacles. This was tested experimentally by subjecting individual animals to varying light intensities, measured by means of a Gossen "Lunasix" light meter, and by interfering with the beam of light by cutting off the source for two seconds. The time of (a) the onset of activity and (b) the duration of the period of activity was noted. Figure 6 shows the results of these experiments and demonstrates that with decreasing light intensity the time of onset of the period of activity after a period in the dark was increased, but that the length of the period of activity remained the same whatever the light intensity (approximately thirty seconds). It was further noted that at high light intensities many more tentacles took part in the beating activity whilst at low intensities the number of participating tentacles decreased. At light intensities below 110 foot candles, no recognizable response was noted. It was finally demonstrated that extension of the dark phase had no effect upon the tentacles; they commenced beating at the same point in time relative to the start of the dark phase as they did after experiencing the dark for only two seconds. It was apparently the onset of darkness, not the termination of darkness, that initiated this activity.

It is possible that the eyes located on the optic tentacles were responsible for detecting the changes in light intensity. A number of bivalves possess pallial eyes.

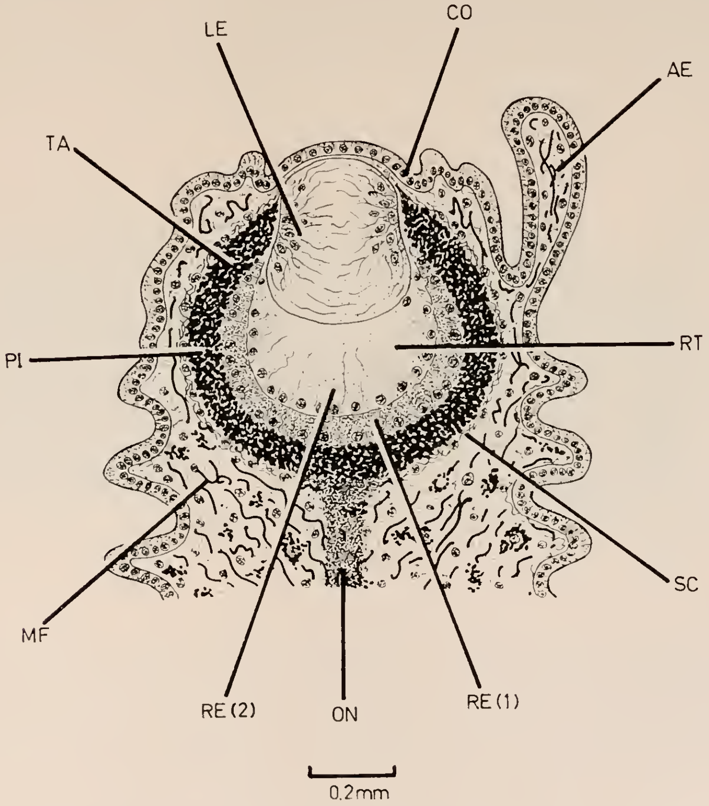

FIGURE 7. *Laternula truncata,* a longitudinal section through an eye located on the tip of an optic tentacle. Abbreviations are: AE, Appendage of the eye; CO, Cornea; LE, Lens; MF, Muscle fibers; ON, Optic nerve; PI, Pigment granules; RE(1), Proximal retina; RE(2), Distal retina; RT, Retinal threads; SC, Sclerotic; TA, Tapetum.

They are perhaps the most well developed in the Pectinacea, *e.g., Pecten* (Dakin, 1910) *Chlamys* and *Amussium* and *Spondylus* (Dakin, 1928). Yonge (1967) has shown that *Pedum spondyloideum* also possesses pallial eyes. In the Cardiacea, the cockle *Cardium edule* possesses numerous simple pallial eyes (Barber and Wright, 1969) while *Tridacna* possesses eye-like structures which Yonge (1936) termed hyaline organs and which were thought to be used for concentrating light upon symbiotic zooxanthellae. Stasek (1966) showed, however, that these structures also possess a sensory function.

Examination of the eyes of *Laternula truncata* in stained section suggests that they are well developed true photoreceptors (Fig. 7). They possess a large lens (LE), lying under a cornea (CO) composed of somewhat flattened cells of the tentacle epithelium. The cup of the eye is composed of three layers which have been named by analogy: (1) An outer sclerotic coat (SC) composed of fibrous material and some muscle fibers. (2) A tapetum (TA) or choroid layer composed of cells containing numerous brown pigment granules (PI). The nuclei of these cells occupy a central or inner peripheral position in the cell. (3) A double retinal layer, in which the proximal layer [Re(1)] is somewhat smaller and darker staining than the distal layer [RE(2)]. The nuclei of these two cell layers are small and basally situated. The nuclei are thus arranged in two distinct lines. From the innermost layer are directed towards the lens a large number of threads (RT) some of which may represent cell boundaries while others may be fine neurofibrils. No positive result was obtained, however, with Holme's stain for nervous tissue except to identify the optic nerve (ON). From the outer border of the optic tentacle arises a fleshy projection of the epithelium, here termed the eye appendage (AE). No function is as yet attributable to this structure.

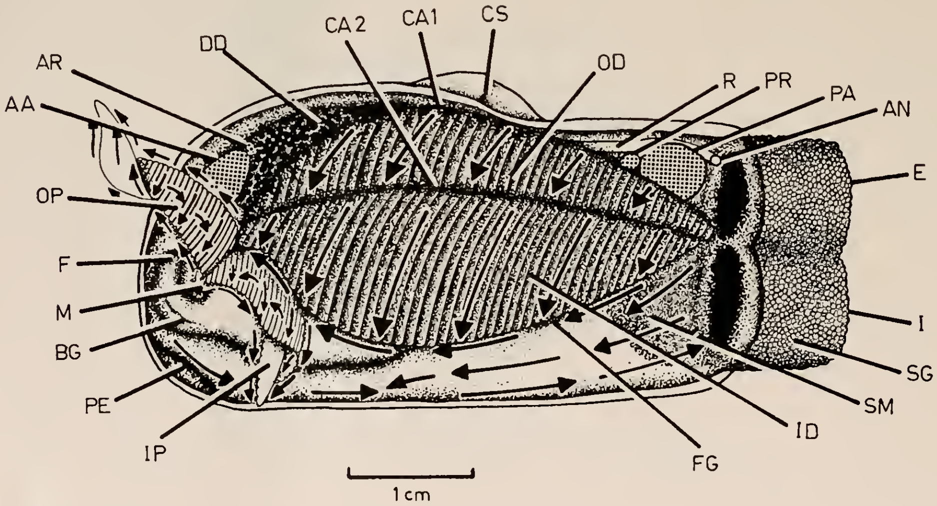

FIGURE 8. *Laternula truncata,* the anatomy and ciliary currents of the organs of the mantle cavity after removal of the left mantle lobe and shell valve. Abbreviations are: AA, Anterior adductor muscle; AN, Anus; AR, Anterior pedal retractor muscle; BG, Byssal groove; CAL, Point of cuticular fusion between the ascending lamella of the outer demibranch and the visceral mass; CA2, Ctenidial axis; CS, Crack in shell; DD, Digestive diverticula; E, Exhalant siphon; F, Foot; FG, Ventral marginal groove; I, Inhalant siphon; ID, Inner demibranch; IP, Inner labial palp; M, Mouth; OD, Outer demibranch; OP, Outer labial palp; PA Posterior adductor muscle; PE, Pedal gape; PR, Pedal retractor muscle; R, Rectum; SG, Sand grains; SM, Siphonal musculature.

The musculature. The anterior and posterior adductor muscles are small (Figs. 1 and 9, AA, PA), the latter somewhat the larger. A similar condition exists in *Clavagella* (Soliman, 1967) and in *Cuspidaria* and *Poromya* (Yonge, 1928). In *Brechites,* the posterior adductor is absent and the anterior adductor is very much reduced (Purchon, 1956). Internally to these two muscles are inserted the anterior (AR) and the posterior (PR) pedal retractors. The latter are very small. These muscles serve to move the foot (F), which in the adult is very small and ineffectual as a digging tool. In *Clavagella,* the foot is very reduced and the pedal retractor muscles absent (Soliman, 1967). The foot of *Laternula truncata* possesses strong ciliary currents and the tip of the foot was often observed between the lips of the mouth, where it may act as an accessory organ for the removal of sediment. A similar habit has been observed in *Dreissena polymorpha* (B. S. Morton, 1969) and *Adula falcata* (Fankboner, 1971).

The foot of *L. truncata* possesses a small byssal groove (Figs. 8 and 9, BG) but there is no functional byssal gland in the adult as there is in *Lyonsia norvegica* (Ansell, 1967) and *Mytilimeria* and *Entodesma* (Yonge, 1952). *Cuspidaria* and *Poromya* (Yonge, 1928) possess a nonfunctional byssal groove.

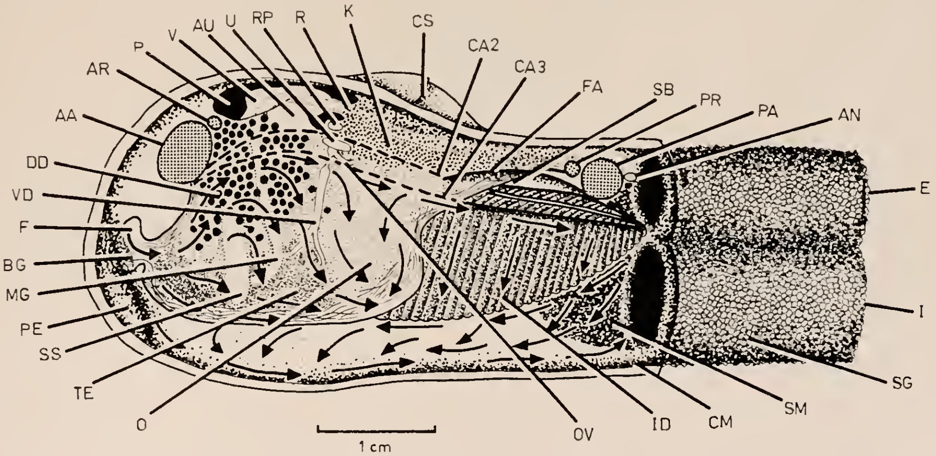

FIGURE 9. *Laternula truncata,* the general anatomy and the ciliary currents of the mantle cavity after removal of the left shell valve, mantle lobe, labial palps and ctenidium. Abbreviations are: AU, Auricle; CA3, Point of cuticular fusion between the ascending lamella of the inner demibranch and the visceral mass; CM, Cut edge of mantle; FA, Free ctenidial axis; K, Kidney; MG, Mid gut; O, Ovary; OV, Oviduct; P, Pericardium; RP, Reno-pericardial aperture; SB, Supra-branchial chambers; SS, Style sac; TE, Testis, U, Urino-genital aperture; V, Ventricle; VD, Vas deferens. (Other abbreviations as in Figure 8.)

The ctenidia and labial palps. The ctenidia of *Laternula truncata* comprise two sub-equal demibranchs of which the inner (Fig. 8, ID) is the longer the outer (OD) being considerably reduced.

The upper margin of the ascending lamella of the outer demibranch (CA1) is attached by cuticular fusion to the visceral mass anteriorly and to the dorsal edge of the mantle posteriorly. Cuticular fusions of this type have already been noted by Atkins (1937b) for *Ensis.* The membranous upper margins of the ascending lamella of the inner demibranchs (Fig. 9, CA3) are attached to the visceral mass by cuticular fusions anteriorly, but posteriorly behind the visceral mass the two demibranchs are united by tissue fusion as in many Leptonacea (Oldfield, 1961; B. S. Morton, 1972). It was noted in some dissected specimens that the marginal membrane of the ascending lamella of the inner demibranch was often separated from the posterior region of the visceral mass. At first it was thought that this separation represented a true aperture on each side of the body, but careful ventral emargination of the shell valves of further specimens and gentle displacement of the ctenidi showed that under natural conditions no aperture exists. Nakazima (1967, 3 pages 149–150) noted a similar condition in *Halicardia* and wrote: "The inner margin of the inner demibranchs is attached to the viscero-pedal mass by means of a membrane with a rather thick edge. . . . The above described attachment of the inner demibranch is assumed to be so delicate as to be separated with a slight strain. It is, therefore, doubtful whether the junction all along the line (*of attachment*) is entire or not." This appears true for *L. truncata* although in this species it is thought that the ctenidial/visceral mass junction is not usually separated even though artificial separation only occurs at one place.

Posteriorly an aperture (Fig. 12, 1A) is present in the ctenidal axis (CA2) of both left and right sides. Further posteriorly, behind the visceral mass these apertures unite to form a large Y-shaped cavity.

The ctenidia are flat, heterohabdic, eulamellabranchiate and plicate; the frontal ciliary currents all beat towards the ventral marginal groove (FG) of the inner demibranch and the ctenidia can thus be ascribed to type E (Atkins, 1937a), a condition which is also present in many of the Tellinidae and Semelidae (Atkins, 1937a; Yonge, 1949) as well as other Anatinacea, *e.g.*, *Brechites penis* (Purchon, 1956) and *Entodesma* and *Mytilimeria* (Yonge, 1952). There is no oralward current in the upper margin of the ascending lamella of either the inner or the outer demibranch.

The number of filaments per plica varied from specimen to specimen. The minimum recorded number from a small animal was 21, while the maximum number recorded was 27. In these two cases there were, respectively, 10 and 13 small filaments on each side of the plica and a single primary filament at the apex. Occassionally two large filaments shared the apex. Inter-filamenter and interlamellar junctions are present in both demibranchs.

These particles arriving at the ventral margin of the inner demibranch within the plical trough pass into the ventral groove. Larger particles arriving at this point on the crests of the plicae do not enter the groove. A similar condition has been reported for *Pandora* (Allen, 1954) and *Brechites* (Purchon, 1956). Large, unacceptable particles are removed either by the rejectory currents of the mantle or visceral mass before reaching the labial palps or are removed by the labial palps themselves and subjected to the resorting mechanism of the inner palp surface.

The ctenidial/labial palp junction is of Category 3 (Stasek, 1963) and is thus similar to a wide variety of bivalves with differing habits and with diverse phylogenetic affinities.

The labial palps (Fig. 8, RP, LP) are very long and fulfill the typical sorting function of most lamellibranchs. Material passing over the crests of the palp ridges to the mouth (M) is accepted while material passing to the tips of the palps

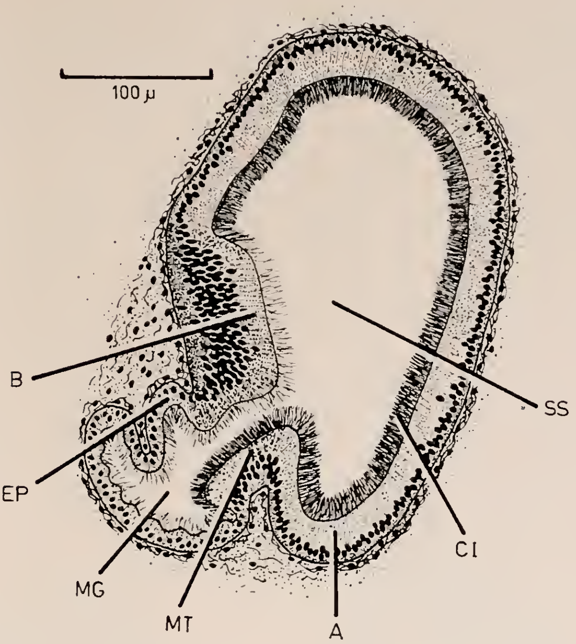

FIGURE 10. *Laternula truncata,* a transverse section through the style sac showing the distribution of the different epithelia. Abbreviations are: A, A cell layer; B, B cell layer; CI, Cilia; EP, Epithelium of mid gut; MG, Mid gut; MT, Minor typhlosole; SS, Style sac.

is rejected and is eventually removed by the rejectory currents of the mantle. Resorting mechanisms for particles of intermediate size also exist on the folds of the labial palps.

The ciliary currents of the mantle and visceral mass. The ciliary currents on the mantle surface serve two major funtcions. They transport particles from the postero-dorsal regions of the mantle cavity toward the labial palps, thereby acting as acceptor tracts (Figs. 8 and 9). Mid ventrally, however, the cilia of the mantle beat in a posterior direction towards the inhalant siphon. Unwanted material so collected is periodically ejected as pseudofeces.

The ciliary currents of the visceral mass are rather more complicated, but their ultimate effect is to pass material to a mid ventral point. Particles arrive at this point from dorsal and anterior directions, those from the latter coming largely from the foot and from recirculatory currents located antero-dorsally on the visceral mass. Particulate material, upon arrival at the mid-ventral terminus either falls onto the rejectory currents of the mantle or is removed by the ctenidial currents to be ultimately rejected by the mantle. Very similar ciliary currents of both the visceral mass and the mantle have been reported for *Cochlodesma praetenue* (Allen, 1958).

The alimentary canal. The stomach of *Laternula truncata* has been studied by Purchon (1958) and designated as belonging to his Type 4. The present investigation has not revealed any new features of stomach morphology which warrant redescription.

The conjoined style sac and mid gut leaves the stomach at the postero-ventral border and passes ventrally into the visceral mass (Fig. 9, SS). The mid gut (MG) then turns upon itself and travels dorsally to pass through the ventricle (V) and the kidney (Fig. 12, R) terminating in the anus (Fig. 9, AN) behind the posterior adductor muscle. In *Cochlodesma* the rectum passes above the kidney (Allen, 1958).

In transverse section (Fig. 10) the style sac can be seen to comprise a number of distinct epithelial regions. Th style sac (SS) itself is bordered by the A and B cell layers. The cells comprising the A cell layer are approximately 18 μ tall with a dense border of cilia 12 μ long. The nuclei of these cells occupy a basal position. The cells of the B cell layer (the major typhosole) are approximately 60 μ tall, with nuclei centrally placed. The sparsely distributed cilia they possess are 15 μ long. The epithelia bordering the gutter connecting the style sac and mid gut (MG) are of two types. The minor typhlosole (MT) is made up of cells 15 μ tall with basal nuclei and short cilia, 10 μ long. The remaining layer comprises the cells of the mid gut which are 6 μ tall with sparsely distributed cilia 10 μ long. The epithelial zones thus recognized in *L. truncata* comply, in general terms with the zones recognized in other lamellibranchs with a conjoined style sac and mid gut, *e.g., Cardium edule* (B. S. Morton, 1970) and *Venerupis philippinarus* (Kato and Kubomura, 1954).

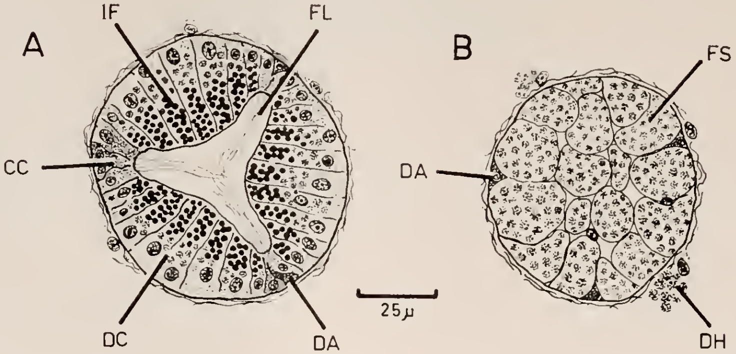

FIGURE 11. *Laternula truncata,* transverse sections through a digestive tubule at (A) the time of absorption and (B) at the time of breakdown. Abbreviations are: CC, Crypt cell; DA, Darkly staining crypt cell; DC, Digestive cell; DH, Digested food material in the haemocoel; FL, Flagella; FS, Fragmentation spherules; IF, Ingested food material.

The digestive diverticula (Fig. 9, DD), surround the stomach and open into the stomach via the left pouch, and via ducts opening directly into the left and right sides of the stomach. The primary and secondary ducts of the diverticula are short and ultimately open into the digestive tubules which bear a very close resemblance to those of tubules which bear a very close resemblance to those of *Cochlodesma* (Allen, 1958) and *Cuspidaria* (Yonge, 1928). The digestive diverticula of different specimens showed a variety of structural conditions. Mature digestive tubules, when seen in transverse section (Fig. 11A) showed that the digestive cells (DC) had ingested very many comparatively large food particles (IF), (usually) 2–3 μ in diameter, but sometimes as large as 5 μ. The crypt cells (CC) possess flagella (FL) 25 μ long. Small pyramid shaped darkly staining cells (DA) in the crypts resemble those thought by Owen (1970) to be secretory in *Cardium*. Similar cells have been observed in *Halicardia* by Nakazima (1927). Another condition encountered in *L. truncata* was of breakdown (Fig. 11B) both of the digestive cells, to produce large fragmentation spherules (FS), and of the flagellated crypt cells. The small darkly-staining cells in the crypts (DA) alone remained apparently intact. Allen (1958) noted the characteristic large distended digestive cells in *Cochlodesma*, but did not appreciate that these cells were probably in the process of breakdown.

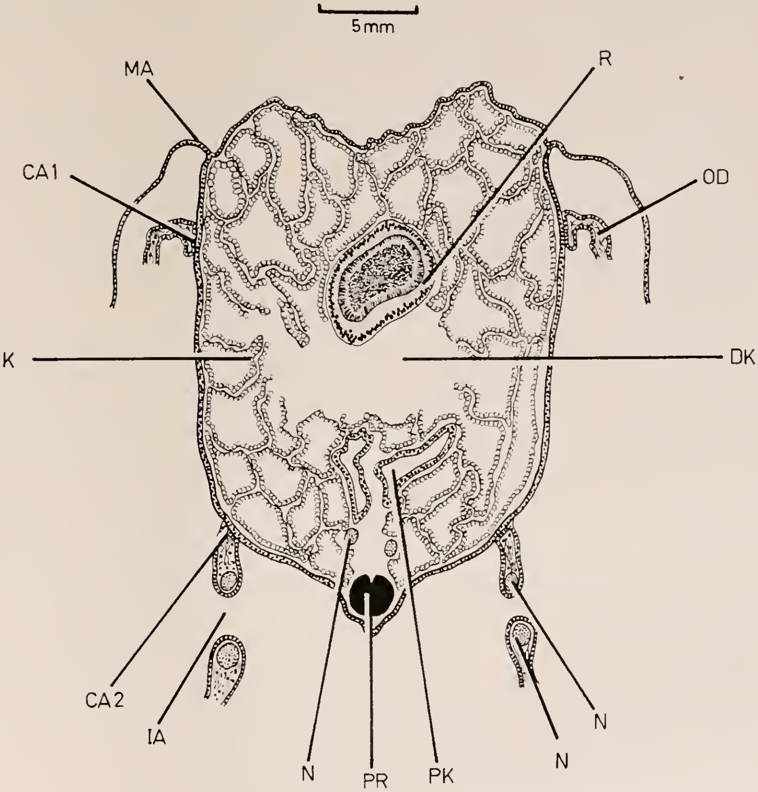

FIGURE 12. *Laternula truncata*, a transverse section through the kidney in the region of the rectum. Abbreviations are: DK, Distal limb of the kidney; IA, Aperture in ctenidial axis; K, Kidney; MA, Mantle; N, Nerve; PK, Proximal limb of the kidney. (Other abbreviations as in Figure 8.)

The epithelium of the secondary duct which, as in *Cochlodesma* (Allen, 1958), characteristically possesses a brush border also breaks down. During this process large numbers of spherical droplets are budded off within fragmentation spherules from the distal surfaces of the cells. Other droplets are released into the hemocoel from the bases of the secondary ducts and the digestive tubules (DH). These bear a very close resemblance to the droplets observed within the cells of the kidney.

The pericardium and kidney. The pericardium (Fig. 9, P) lies anterior to the umbo and contains the ventricle (V), which is penetrated by the rectum (R), and two auricles. From the posterior wall of the pericardium a single renopericardial aperture (RP) opens into the kidney (K). The duct leading into the kidney from this aperture is, for a short distance, single, but soon divides into two proximal limbs (Fig. 12, PK). These proximal limbs are extensively ciliated at their origin, but subsequently the cilia are lost and the lumina of the tube increases in size (Fig. 12, PK). The proximal limbs are surrounded by, and posteriorly communicate with the fused lumina of the distal limbs (Fig. 12, DK) of the kidney. The distal limbs communicate with the suprabranchial chamber on each side of the body via a renal aperture which opens into the urino-genital papilla (Fig. 9, U).

The walls of the distal limbs only of the kidney comprise a system of tubules which open into the distal limbs. The cells comprising the tubules possess in different specimens differing amounts of spherical concretions in the free end of the cell. In some specimens the amount was small while in others it was very large.

It has been shown that the pericardial gland of *Dreissena polymorpha* (B. S. Morton, 1969) possesses varying amounts of solid material at different times in relation to the time of feeding. Similar observations have been made by Kowalewsky (1889).

The reproductive system. As in all Anomalodesmata that have been studied, *e.g., Brechites* (Purchon, 1956; 1960), *Laternula truncata* is an hermaphrodite. The testis occupies a ventral position in the visceral mass (Fig. 9, TE) and from each side of the body arises a vas deferens (VD) which passes postero-dorsally. Just before opening into the urino-genital aperture a constriction of the duct occurs, and thereafter the border of the duct is heavily ciliated.

The ovary occupies a dorsal position in the visceral mass (O) and from each side of the body a short oviduct (OV) arises which quickly fuses with the vas deferens. The product of this fusion is a short hermaphrodite duct which opens via a slit-like urino-genital aperture (U) into the supra-branchial chamber. Strong cilia surround the lips of the urino-genital aperture and beat outwards setting up a strong current of water. In all of the Anomalodesmata hitherto studied, *e.g., Cochlodesma* (Allen, 1958) and *Entodesma* and *Mytilimeria* (Yonge, 1952) the gonads open by separate ducts, which also open separately from the renal aperture. The condition existing in *L. truncata* is therefore unusual.

DISCUSSION

The Anomalodesmata comprise a diverse assemblage of little known bivalves. The most well known superfamily, the Pandoracea of which the Laternulidae are a family, are all adapted to a sedentary mode of life living intertidally or sublittorally in sandy deposits. Some of them, *e.g., Lyonsia* (Ansell, 1967) and *Entodesma* (Yonge, 1952) possess a functional byssal gland. This organ, in adult, *Laternula* is reduced to a non-functional byssal groove. In the Poromyacea, *Cuspidaria* and *Poromya* also possess non-functional byssal grooves (Yonge, 1928). In the adult *Laternula truncata* the reduced foot is probably used as an accessory mantle cleansing organ, although in the young animal it is used for burrowing.

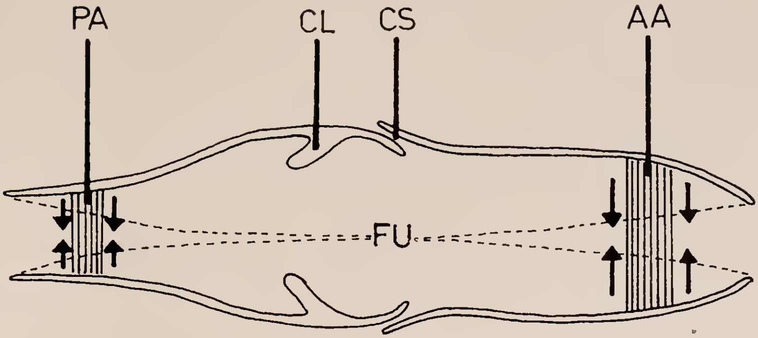

FIGURE 13. *Laternula truncata,* a horizontal section through the shell to demonstrate the method of movement of the shell valves. Abbreviations are: AA, Anterior adductor muscle; CL, Clavicle; CS, Crack in shell; FU, Fulcrum; PA, Posterior adductor muscle.

The microstructure of the shell of the Pandoracea is most similar to that of the Poromyacea (Taylor *et al.,* 1972). The thinness of the shell in *L. truncata* in particular and in the Pandoracea in general (Taylor, *et al.,* 1972) possibly represents another step in the trend of this group towards a sedentary mode of life. The thinness of the shell in *L. truncata* has necessitated the development of a clavicle or strengthening strut for each shell valve. Such a system is not designed to protect the animal from external damage, but from the internal compression forces created by the adductor muscles. A further modification has been the development of a lithodesma which effectively fixes the ligament, thereby preventing the shell valves from opening under the pressure exerted principally by the primary ligament and by the secondary ligament of fused periostracum. The secondary ligament is also found in other genera of the Pandoracea, *e.g., Pandora* and *Cochlodesma* (Allen, 1954; 1958) and *Entodesma* and *Mytilimeria* (Yonge, 1952) although only in the latter two genera is there a lithodesma. A lithodesma occurs in *Mydora, Myochama* and *Chamostrea* (Thiele, 1935), and in *Thracia* (Tebble, 1966). The trend in the Pandoracea towards the relative immobilization of the ligament by means of a lithodesma has resulted in *L. truncata* in the adductor muscles being relatively small and ineffectual. This is especially true of the posterior adductor. The pedal retractors of the adult are similarly very small.

It would thus seem that shell movements in *Laternula* (Fig. 13) are small and are brought about by the contraction of (1) the anterior and posterior adductor muscles (AA, PA) and (2) the orbital muscles acting against the fulcrum (FU) formed by the meeting of the shell valves dorsally and ventrally in the mid line only. The muscles act in opposition, not as in the typical bivalve to the ligament but to the flexibility of the shell itself. The flexibility of the shell is enhanced by the median crack (CS) in each of the shell valves which allows this action to occur, without putting excessive strain upon the fragile shell. The clavicles (CL) give added strength to the shell. Significantly, *Cochlodesma praetenue* also possesses a crack in the shell (Tebble, 1966).

It is envisaged that this trend towards the fixation of the shell in the Laternulidae has proceeded further in the Clavagellacea (Soliman, 1967; Purchon, 1956) and has resulted in the total fixation of the shell by the development of an adventitious shell and the adoption of muscular pumping as a means of burrowing and possibly of collecting food material. Significantly the muscles which perform this action are greatly enlarged orbital muscles (Purchon, 1960). There is only a small anterior adductor muscle remaining in *Brechites* (Purchon, 1958) and significantly the shell valves are free when young (Taylor *et al.,* 1972). In the Saxicavacea (Yonge, 1971) also, the greatly enlarged orbital muscles are used in the movement of the shell valves.

The siphons of *L. truncata* possess numerous tactile tentacles and nine optic tentacles. The optic tentacles have been shown to possess true eyes. Such true eyes are unusual, especially since well developed eyes are usually only found [exceptions being *Pedum spondyloideum* (Yonge, 1967) and *Spondylus* (Dakin, 1928)] in free living bivalves, *e.g.,* the Pectinacea (Dakin, 1910, 1928). Charles (1966) has stated that the eyes of the Bivalvia cannot be related to any development from a basic plan, as can most of the gastropod eyes. The eyes of *Cardium* (Barber and Wright, 1969) are very simple and do not even possess a lens. The eyes of *L. truncata* on the other hand, are possibly as advanced as those of the Pectinacea, especially since there is a double retina, as in *Pecten* (Schlicher, 1926). In other respects also the eyes of the two animals are very similar and the sheet-like layers of the lens of *L. truncata* may suggest an even greater refinement in this species.

In *L. truncata,* the eyes detect subtle changes in light intensity and it is thought that they constitute the receptive portion of a reflex arc the efferent response of which is the curious flicking motions of the tactile tentacles. The shadow reflex recorded for *L. truncata* can be equated with the primary "off" reponse demonstrated for *Cardium edule* (Barber and Land, 1967), *Pecten* (Land, 1966) and *Onchidium* (Fujimoto, Yanase, Okumo and Iwata, 1966) and coincidental with a fall in light intensity. Such a response would strongly suggest therefore that the distal retina of the eye of *L. truncata,* at least, is a ciliary photoreceptive structure since an "off" response is not associated with a rhabdomal receptor cell. This "shadow" reflex noted for example by Sharp (1883) in *Solen* has subsequently been observed in other bivalves, *e.g., Spisula* (Kennedy, 1960) and *Tridacna* (Stasek, 1965) and in most cases resulted in withdrawal of the siphon. It must therefore be regarded as constituting a defensive reflex in most bivalves.

It is thought that the flicking of the tactile tentacles of *L. truncata* may serve a number of functions. The tentacles flick just prior to siphonal withdrawal, an action which perhaps consitutes a defensive response, concealing the outline of the sand covered siphons by bringing other particles over them. A comparable behavior pattern occurs in certain flatfish when they settle on the surface of the sand, by flicking sand particles over them to break up their outline. The flicking action of the tentacles in *L. truncata* just before the siphons reopen in all probability clears the sand away prior to the emergence of the siphons and the resumption of feeding. The "shadow" reflex is unusual, however, since it is accompanied by closure of the siphonal aperture but not by siphonal withdrawal and cannot thus be regarded as a defensive reflex. To the contrary such an action would only serve to attract the attention of predators. The resting tentacles are cryptically covered and the siphonal covering of adhered sand grains make the animal impossible to see, at least to the human eye, in its natural environment. Mangroves are characteristically the feeding grounds of a large number of permanent and migratory wading birds. Such animals wade along the shoreline seeking food. A quiescent and camouflaged *L. truncata* would possibly escape detection, but would cleanse itself of the disturbed sediments kicked up by the bird once this (and its shadow) had passed. Perhaps too it capitalizes on the detritus kicked up by the bird. Such a feeding mechanism, though unproven, is a distinct possibility. *L. truncata* may thus be regarded as a specilized detritus/suspension feeder, collecting material raised into suspension as a result of extraneous disturbance. Within the mantle cavity the organs concerned with the collection, sorting and either acceptance or rejection of this material are well developed. The ctenidia are plicate, passing food material into the ventral marginal food groove of the inner demibranch only. The labial palps of many of the deposit feeding Tellinacea, *e.g., Abra alba* (Yonge, 1949) and the rejectory tracts of the mantle and visceral mass are efficient. The foot probably acts as an accessory cleansing organ. The organs of the mantle cavity are thus typical of deposit feeding bivalves.

The digestive tubules of *Laternula* are similar to those of *Cochlodesma* (Allen, 1958) and *Cuspidaria* (Yonge, 1928). It has been demonstrated for *Laternula kamakurana* by Nakazima (1955) and in this study of *L. truncata* that the digestive cells are capable of ingesting comparatively large particles, *e.g.,* (2–5 μ), as in *Cuspidaria* (Yonge, 1928). Characteristically small particles only are ingested by the majority of bivalves; *Teredo* (Yonge, 1926) and *Nuculana* (Yonge, 1939) being exceptions in the possession of "specialized" diverticula for the ingestion of comparatively large particles. According to Owen (1956) the digestive diverticula of the Nuculidae are not absorptive but are secretory in function.

L. truncata is a simultaneous hermaphrodite, as apparently are all other members of the Pandoracea and Clavagellacea. Unlike the other Pandoracea, however, the testis, ovary and the excretory aperture of *L. truncata* open into a common urinogenital papilla. Such a modification, if one accepts that the hermaphroditic condition is characteristic of specialized bivalves as suggested by Purchon (1968), must represent an even greater specialization in this species.

Taylor, *et al.* (1972) suggest that the Clavagellacea and Poromyacea have a close affinity with the Pandoracea, and further suggest that all of these lineages arise from a Pholadomyacean stock in the Trias or lower Jurassic. Runnegar (1972) has attempted to elucidate the morphology of *Pholadomya candida* based upon an examination of the shell. There can be little doubt that it would be highly profitable to examine a living *Pholadomya* and perhaps so to shed some light on the evolution of the Anomalodesmata. The morphological eccentricities of *L. truncata,* though largely of a specialized nature and highlighting the success of the Pandoracea in general also seem to foreshadow the extreme morphological specialization of the Clavagellacea.

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SUMMARY

Laternula truncata (Lamarck) is a highly specialized member of a somewhat diverse assemblage of bivalves, the Anomalodesmata. It is found buried in coarse sand on the seaward side of mangrove formations in South East Asia. (1) The shell is thin and possesses a lithodesma, which effectively prevents the opening of the shell in the typical bivalve manner. Movement of the shell is achieved by the bending of the dorsal margin of the shell at a dorso-ventral crack in each shell valve and by the flexibility of the shell itself. Ventral orbital muscles are probably as important in valve movements as are the reduced adductors. A strengthening strut or clavicale is present on each valve. (2) Mantle fusions are of type B ventrally, and type C dorsally. There is a small pedal gape, but no fourth pallial aperture. (3) The siphons which are of type C, are covered in sand grains. At their tips they possess tactile tentacles and optic tentacles; the structure of the complex eyes which the latter possess are described. The tactile tentacles undergo unusual flicking motions when a shadow falls over the siphons. This may be a defensive and a cleansing response and also play a role in feeding. (4) The ctenidia are plicate and heterorhabdic and have ciliary currents of type E (Atkins, 1937a). The outer demibranch is reduced, and the ctenidial axis possesses an aperture on each side of the body which unite posteriorly to form a Y-shaped cavity. (5) The ctenidial/labial junction is of type 3 (Stasek, 1963), and the labial palps are large. (6) The stomach is of type 4 (Purchon, 1958). The structure of the style sac and the digestive diverticula are described. (7) The structure of the kidney is described. (8) The reproductive system is described. *L. truncata* is hermaphrodite and the oviduct, vas deferens and urinary duct open, unusually, via a common urinogenital aperture. Many of the morphological adaptations of *L. truncata* foreshadow the highly specialized condition in the Clavagellacea.

LITERATURE CITED

ALLEN, J. A., 1954. On the structure and adaptations of *Pandora inaequivalvis* and *P. pinna*. *Quart. J. Microscop. Sci.*, 95: 473–482.

ALLEN, J. A., 1958. Observations on *Cochlodesma praetenue* (Pulteney) (Eulamellibranchia). *J. Mar. Biol. Ass. U.K.*, 37: 97–112.

ALLEN, J. A., 1960. The ligament of *Cochlodesma praetenue* (Pulteney). *J. Mar. Biol. Ass. U.K.*, 39: 445–447.

ANSELL, A. D., 1967. Burrowing in *Lyonsia norvegica* (Gmelin) (Bivalvia: Lyonsidae). *Proc. Malacol. Soc. London*, 37: 387–393.

ATKINS, D., 1937a. On the ciliary mechanisms and interrelationships of lamellibranchs. Part III. Types of Lamellibranch gills and their food currents. *Quart. J. Microscop. Sci.*, 79: 375–421.

Atkins, D., 1937b. On the ciliary mechanisms and interrelationships of lamellibranchs. Part IV. Cuticular fusion, with special reference to the fourth aperture in certain lamellibranchs. *Quart. J. Microscop. Sci.,* **79**: 423–445.

Barber, V. C., and M. F. Land, 1967. Eye of the cockle *Cardium edule:* anatomical and physiological investigation. *Experientia,* **23**: 677–678.

Barber, V. C., and D. E. Wright, 1969. The fine structure of the eye and optic tentacle of the mollusc *Cardium edule. J. Ultrastruct. Res.,* **26**: 515–528.

Beedham, G. E., 1958. Observations on the non-calcareous component of the shell of the Lamellibranchia. *Quart. J. Microscop. Sci.,* **99**: 341–357.

Carleton, H. M., and R. A. B. Drury, 1957. *Histological Techniques.* Oxford University Press, Oxford, 442 pp.

Charles, G. H., 1966. Sense organs (less cephalopods). Pages 455–521 in K. M. Wilbur and C. M. Yonge, Eds., *Physiology of the Mollusca,* Vol. II. Academic Press, New York and London.

Dakin, W. J., 1910. The eye of *Pecten. Quart. J. Microscop. Sci.,* **55**: 49–112.

Dakin, W. J., 1928. The anatomy and physiology of *Spondylus,* with a particular reference to the lamellibranch nervous system. *Proc. Roy. Soc. London, Series B.,* **103**: 337–354.

Douvillé, H., 1912. Classification des Lamellibranches. *Bull. Soc. Géol. France,* **12**: 419–467.

Fankboner, P. V., 1971. The ciliary currents associated with feeding, digestion, and sediment removal in *Adula (Botula) falcata* Gould 1851. *Biol. Bull.,* **40**: 28–45.

Fujimoto, K., T. Yanase, Y. Okumo and K. Iwata, 1966. Electrical responses in the *Onchidium* eye. *Mem. Osaka, Gakugei Univ.,* **B15**: 98.

Kato, K., and K. Kubomura, 1954. On the origins of the crystalline style of lamellibranchs. *Sci. Rep. Saitama Univ.,* **B3**: 135–152.

Kennedy, D., 1960. Neural photoreceptors in a lamellibranch mollusc. *J. Gen. Physiol.,* **44**: 277–279.

Kira, T., 1962. *Shells of the Western Pacific in Color,* Vol. I. Hoikusha, Osaka, Japan, 224 p.

Kowalewsky, A., 1889. Beitrag zur Kenntnis der Exkretionsorgane. Mollusken. *Biol. Zentralbl.,* **9**: 66–70.

Land, M. F., 1966. Activity in the optic nerve of *Pecten maximus* in response to changes in light intensity and to pattern movement in the optical environment. *J. Exp. Biol.,* **45**: 83–99.

Macnae, W., 1968. A general account of the fauna and flora of mangrove swamps and forests in the Indo-West-Pacific region. Pages 73–270 in F. S. Russell and C. M. Yonge, Eds., *Advances in Marine Biology,* Vol. 6. Academic Press, London and New York.

Morton, B. S., 1969. Studies on the biology of *Dreissena polymorpha* Pall. 1. General anatomy and morphology. *Proc. Malcol. Soc. London,* **38**: 301–321.

Norton, B. S., 1970. The tidal rhythm and rhythm of feeding and digestion in *Cardium edule J. Mar. Biol. Ass. U.K.,* **50**: 499–512.

Morton, B. S., 1972. Some aspects of the functional morphology and biology of *Pseudopythina subsinuata* (Lischke) (Bivalvia; Leptonacea) commensal on stomatopod crustaceans. *J. Zool.,* **166**: 79–96.

Morton, B. S., 1973a. Some aspects of the biology and functional morphology of the organs of feeding and digestion of *Limnoperna fortunei* (Dunker) (Bivalvia: Mytilacea). *Malacologia,* in press.

Morton, B. S., 1973b. The biology and functional morphology of *Galeomma (Paralepida) takii* (Kuroda) (Bivalvia: Leptonacea). *J. Zool.,* **169**: 133–150.

Morton, J. E., 1967. *Molluscs.* Hutchinson University Library, London, 244 pp.

Nakazima, M., 1955. On the structure and function of the midgut gland of Mollusca, with a general consideration of the feeding habits and systematic relations. *Jap. J. Zool.,* **11**: 469–566.

Nakazima, M., 1967. Some observations on the soft parts of *Halicardia nipponensis* Okutani. *Venus,* **25**: 147–160.

Newell, N. D., 1965. Classification of the Bivalvia. *American Museum Novitates,* **2206**: 1–25.

Oldfield, E., 1961. The functional morphology of *Kellia suborbicularis* (Montagu), *Montacuta ferruginosa* (Montagu) and *M. substriata* (Montagu) (Mollusca, Lamellibranchiata). *Proc. Malacol. Soc. London,* **34**: 225–298.

Owen, G., 1956. Observations on the stomach and digestive diverticula of the Lamellibranchia. II. The Nuculidae. *Quart. J. Microscop. Sci.*, **97**: 541–567.

Owen, G., 1959. The ligament and digestive system in the taxodont bivalves. *Proc. Malacol. Soc. London*, **33**: 215–224.

Owen, G., 1970. The fine structure of the digestive tubules of the marine bivalve *Cardium edule*. *Phil. Trans. Roy. Soc. London Series B*, **258**: 245–260.

Owen, G., E. R. Trueman and C. M. Yonge, 1953. The ligament in the Lamellibranchia. *Nature*, **171**: 73.

Pelseneer, P., 1911. Les lamellibranches de l'expedition du Siboga. Partie anatomique. *Siboga Exped. Monogr.*, No. **53a**: 1–126.

Purchon, R. D., 1956. A note on the biology of *Brechites penis* (L.). *J. Linn. Soc. Zool.*, **43**: 43–54.

Purchon, R. D., 1958. The stomach in the Eulamellibranchia; Stomach Type IV. *Proc. Zool. Soc. London*, **131**: 487–525.

Purchon, R. D., 1959. Phylogenetic classification of the Lamellibranchia with special reference to the Protobranchia. *Proc. Malacol. Soc. London*, **33**: 224–230.

Purchon, R. D., 1960. A further note on the biology of *Brechites penis* (L.). *Lamellibranchia. Proc. Malacol. Soc. London*, **34**: 19–23.

Purchon, R. D., 1962. Phylogenetic classification of the Bivalvia, with special reference to the Septibranchia. *Proc. Malacol. Soc. London*, **35**: 71–80.

Purchon, R. D., 1968. *The Biology of the Molluusca. International Series of Monographs in Pure and Applied Biology, Zoology Division*, Vol. 40. Pergamon Press, Oxford, 560 pp.

Ridewood, W. G., 1903. On the structure of the gills of the Lamellibranchia.. *Phil. Trans. Roy. Soc. London Series B*, **195**: 147–284.

Runnegar, B., 1972. Anatomy of *Pholadomya candida* (Bivalvia) and the origin of the Pholadomyidae. *Proc. Malacol. Soc. London*, **40**: 45–58.

Russell Hunter, W., 1949. The structure and behavior of *Hiatella gallicana* (Lamarck) and *H. arctica* (L.) with special reference to the boring habit. *Proc. Roy. Soc. Edinburgh Series B.*, **72**: 271–289.

Schlicher, J., 1926. Das Tapetum im Auge von *Pecten*. *Verh. Natur. Verein Bonn*, **82**: 197–206.

Sharp, B., 1883. On visual organs in *Solen*. *Proc. Acad. Nat. Sci. Philadelphia*, **58**: 248–249.

Soliman, G. N., 1971. On a new clavagellid bivalve from the Red Sea. *Proc. Malacol. Soc. London*, **39**: 389–397.

Stasek, C. R., 1963. Synopsis and discussion of the association of ctenidia and labial palps in the bivalved molluscs. *Veliger*, **6**: 91–97.

Stasek, C. R., 1965. Behavioral adaptation of the giant clam *Tridacna maxima* to the presence of grazing fishes. *Veliger*, **8**: 29–35.

Stasek, C. R., 1966. The eye of the giant clam (*Tridacna maxima*), *Occ. Pap. Calif., Acad. Sci.*, **58**: 1–9.

Taylor, J. D., W. J. Kennedy, and A. Hall., 1972. The shell structure and mineralogy of the Bivalvia. Part 2, Chamacea-Poromyacea, Conclusions. *Bull. Br. Mus. Nat. Hist. Zool.*, in press.

Tebble, N., 1966. *British Bivalve Sea shells*. The British Museum (Natural History), London, 212 pp.

Thiele, J., 1935. *Handbuch der systematischen Weichtierkunde*, Vol. 2, Jena, Germany, 1,022 pp.

Vohra, F. C., 1971. Zonation on a tropical sandy shore. *J. Anim. Ecol.*, **40**: 679–705.

Yonge, C. M., 1926. The digestive diverticula in the Lamellibranchia. *Trans. Roy. Soc. Edinburgh*, **54**: 703–718.

Yonge, C. M., 1928. Structure and function of the organs of feeding and digestion in the septibranchs, *Cuspidaria* and *Poromya*. *Phil. Trans. Roy. Soc. London Series B.*, **216**: 221–263.

Yonge, C. M., 1936. Mode of life, feeding, digestion and symbiosis with zooxanthellae in the Tridacnidae. *Sci. Rep. Gr. Barrier Reef Exped.*, **1**: 283–321.

Yonge, C. M., 1937. The formation of siphonal openings by *Thracia pubescens*. *Proc. Malacol. Soc. London*, **22**: 337–338.

YONGE, C. M., 1939. The protobranchiate Mollusca; a functional interpretation of their structure and function. *Phil. Trans. Roy Soc. London Series B,* **230**: 79–147.

YONGE, C. M., 1949. On the structure and adaptations of the Tellinacea, deposit-feeding Eulamellibranchia. *Phil. Trans. Roy. Soc. London Series B,* **234**: 29–76.

YONGE, C. M., 1952. Studies on Pacific Coast mollusks. V. Structure and adaptation in *Entodesma saxicola* (Baird) and *Mytilimeria nuttallii* Conrad. *Univ. Calif. Publ. Zool.,* **55**: 439–450.

YONGE, C. M., 1957. Mantle fusions in the Lamellibranchia. *Publ. Staz. Zool. Napoli,* **29**: 151–171.

YONGE, C. M., 1959. The status of the Protobranchia in the bivalve Mollusca. *Proc. Malacol. Soc. London,* **33**: 210–214.

YONGE, C. M., 1967. Observations on *Pedum spondyloideum* (Chemnitz) Gmelin, a scallop associated with reef-building corals. *Proc. Malacol. Soc. London,* **37**: 311–323.

YONGE, C. M., 1971. On the functional morphology and adaptive radiation in the bivalve Superfamily Saxicavacea (*Hiatella* (=*Saxicava*), *Saxicavella, Panomya, Panope, Cyrtodaria*). *Malacologia,* **11**: 1–44.

Reference: *Biol. Bull.*, **145**: 532–547. (December, 1973)

ELECTRICAL ACTIVITIES OF THE ANTHOMEDUSAN, *SPIROCODON SALTATRIX* (TILESIUS)

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The functional organization of the nervous system in cnidarians has been studied mainly from the behavioral aspect (for review, see Bullock, 1965). The electrophysiological approach within the past decade has revealed a variety of types of pulses originating in nerves, muscles and non-nervous epithelia which function in a coordinated way for locomotory (Passano and McCullough, 1964; Mackie and Passano, 1968), feeding (Passano and McCullough, 1964, 1965; Mackie, 1968; Rushforth and Burke, 1971) and luminescent behavior (Morin and Cooke, 1971). The potentials recorded from hydrozoans typically show a long time course and are recordable over a wide area with little regional localization.

Some hydroids respond to light or shadow by reflex movements (Kikuchi, 1947; Passano, Mackie and Pavans de Ceccatty, 1967), by changing rhythmic activities (Passano and McCullough, 1962, 1964), by spawning (Yoshida, 1959). These responses are induced by dermal photoreception (Passano and McCullough, 1964) or mediated through the ocelli (Hisada, 1956; Passano, *et al.*, 1967), whose sensory cells conform electron microscopically with the photoreceptive structures of the ciliary type (Eakin and Westfall, 1962) and have neural connections with the outer nerve ring (Jha and Mackie, 1967; Mackie, 1971). Electrical activities associated with photic responses have been recorded in the dermal receptive system of *Hydra* (Passano and McCullough, 1964). Ocelli have been studied biochemically by techniques for pigment analysis (Yoshida, Ohtsuki and Suguri, 1967; Yoshida, 1969) but there have been no published investigations on the electrical activities of hydromedusae evoked by light-off.

The work reported below will deal with electrical activities, especially with those around the nerve ring of the hydromedusan *Spirocodon saltatrix,* a genus which is closely related to *Polyorchis.* As shown in Figure 1, the ocelli of *Spirocodon* are situated on the abaxial surface of each tentacular base and according to Uchida (1927), the first tentacles are formed in each perradius (Fig. 1A, center) and the next in each interradius (Fig. 1A, left and right ends). The number increases by the formation of subsequent tentacles on either side and slightly below those already formed. Thus, in fully grown medusa, the ocelli come to form an arch with the oldest one at its apex. At the same time the tissue of the radial streaks (Fig. 1, Rd S) extends, connecting the ocelli with the marginal ring structures. It is quite possible therefore that conduction pathways between the ocelli and the tentacles on the one hand and the nerve rings on the other are established across the intervening expanse of tentacular tissue (the subtentacular region), over a distance of 3–5 mm in most specimens, up to 8 mm in the largest one. This preparation is uniquely suitable for analysis of events occurring between the periphery (tentacles and ocelli) and the center (nerve ring).

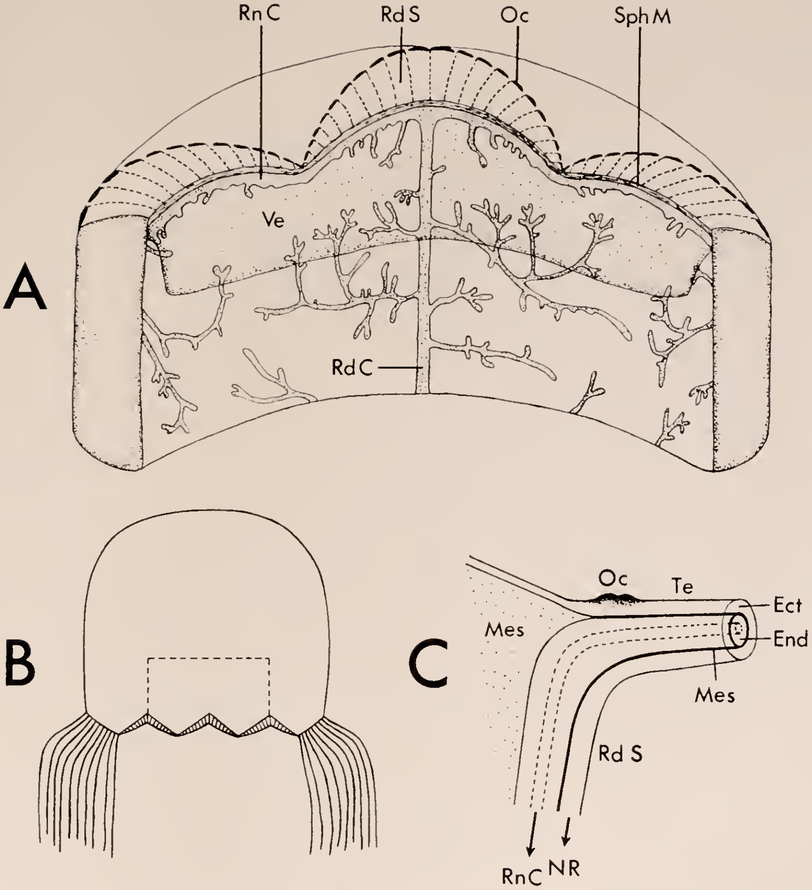

FIGURE 1. Semi-diagrammatic sketches of *Spirocodon saltatrix.* A is a part of the umbrella obtained by cutting along the broken line in B. In A, the velum is shown lying across the subumbrellar surface. The two nerve rings, the inner and the outer, which run circularly along the velar base, are omitted in order to avoid undue complexity. In B, the tentacles of the middle part are cut out. C shows a longitudinal section through the radial streak. Abbreviations used are: Ect, ectoderm; End, endoderm; Mes, mesogloea; NR, nerve ring; Oc, ocellus; Rd C, radial canal; Rd S, radial streak; Rn C, ring canal; Sph M, sphincter muscle; Te, tentacle; Ve, velum. For explanation, see text.

We have found various types of pulses, some possibly neural (narrower in pulse duration than any reported for hydroids) and some myonal, which occur both spontaneously and in response to electrical and photic stimulation. For reader's convenience, abbreviations and characteristics of the pulses recorded are tabulated in Table I.

METHODS

Specimens of the anthomedusa *Spirocodon saltatrix,* 5–7 cm in bell diameter, were collected from the Seto Inland Sea and kept in running sea water in a

TABLE I

Names, abbreviations and characteristics of pulses recorded from Spirocodon saltatrix

Name	Abbreviation	Figure	Characteristics				
			Pulse duration (msec)	Amplitude (mV)	Conduction velocity (cm/sec)	Possible source	Remarks
Marginal pulse	nMP	2A, B	5–10	0.3–1.5	71	Outer nerve ring	Coordinated with QSCP
Pre-swim pulse	PSP	3, dots	3–5	Less than 0.5	125	Outer and inner nerve ring	Trigger VSP and SSP indirectly
Quick and slow compound pulse	QSCP	4A, F	30–100	0.3–1	63*	Quick phase, possibly nerve	Coordinated with nMP
Slow monophasic pulse	SMP	4D, J	50–100	0.3–1	?	?	May conduct locally
Velar swim pulse	VSP	6A, B	250–500	1–4	?	Circular muscle of the velum	Consisted of two phases intervened by a steady phase
Subumbrellar swim pulse	SSP	6C, D	200–500	0.5–2	Circularly, 18 Radially, 6.6	Subumbrellar muscle	Consisted of two phases intervened by a steady phase
Epithelial pulse	EP	8A, B	15–30	0.4–1	12	Exumbrellar epithelium	—
Endodermal pulse	EDP	8D	200–400	1–5	?	Subumbrellar endoderm	Abolished by repetitive stimuli

* The figure was obtained only by one experiment.

laboratory tank. Figure 1A shows a portion of the umbrella which was removed from the area indicated by the broken line in Figure 1B. The tentacles were cut off distal to the ocelli, which are carried on the upper sides of the tentacles near their points of attachment to the exumbrella. Such a preparation contains on the subumbrellar side one radial canal (Rd C) at the center, a part of the ring canal (Rn C), sphincter muscle (Sph M) and velum (Ve), and on the exumbrellar surface one ocellar arch at the center with two halves of it on either side. The type of preparation was modified according to the experimental need and in some cases, smaller preparations having a half of the ocellar arch were employed.

The preparation was placed subumbrellar side up on a silver plate which served as the indifferent electrode and wetted from time to time by dropping sea water. A recording electrode was lowered vertically on to the preparation by means of micro-manipulator. For inspection, polarized light was admitted from below through a hole made in the center of the silver plate. The nerve ring is hard to see without staining, but its position can be estimated fairly accurately with the help of the strong birefringence of the adjacent sphincter muscle.

For photo-stimulation, light from a tungsten lamp (6V, 30W) was directed obliquely on to the preparation through a camera shutter.

Electrical stimuli were delivered through a pair of silver wires led from an isolator (Nihon Kohden, MSE-JM). Square pulses whose durations and amplitudes were in the range of 0.5–5 msec and 3–30V, respectively, were delivered to appropriate sites according to the experimental requirements.

Electrolytically polished stainless steel insect pins were used as recording electrodes. In some cases, the electrodes were connected to a coiled piece of 100 μ silver wire. Floating electrodes of this type were not dislodged by movements caused by vigorous muscle contractions.

Electrical response was recorded by series of electrical instruments manufactured by Nihon Kohden. Amplification was achieved by means of a direct coupled pre-amplifier (MZ-3B) and a main amplifier (AVH-2) with a long time constant. A condenser of 0.01 μF was connected in parallel with the input of the amplifier as an eraser of high frequency noise due to the pre-amplifier. The use of the condenser caused little change in the waveform of recorded potentials. For simultaneous recordings from two sites, another set of input box (AVB-JA) and a condenser coupled amplifier (AVB-2) with time constant of 0.3 sec were used. All the responses were displayed on a dual-beam cathode ray oscilloscope (VC-7A) and photographed by means of a continuous recording camera (PC-1B) from another oscilloscope (VC-MA-7).

RESULTS

Pulses recorded from around the nerve ring

When the recording electrode was placed at points around the nerve ring, two types of quick pulse were recorded spontaneously or in response to electrical stimuli. One of them showed some of the characteristics of the events which Mackie and Passano (1968) termed "marginal pulses (MP's)" and for the reason to be described below we assumed that the pulses represent a predominantly nervous component of the MP's (nMP's). The other type of pulse was biphasic

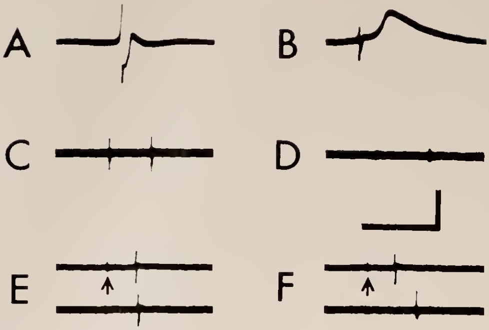

FIGURE 2. Pulses recorded from the outer nerve ring. A and B are nMP's shown by fast sweep speeds. Note a notch in A and a slow positive deflection after the nMP in B. C and D are records obtained from the isolated velum with the nerve ring. E and F are simultaneous recordings of nMP's evoked by electrical stimulation (arrows) on the outer nerve ring. Vertical bar shows 1 mV. Horizontal bar shows: 23 msec in A; 46 msec in B; 100 msec in C-F. Upward deflection is positive in this figure and also in the following figures.

events with amplitudes smaller than nMP's and they always preceded swimming contractions of the velum and the subumbrellar muscle. The term "pre-swim pulse (PSP)" therefore appears to be appropriate.

nMP's were generally triphasic with a small rebound and often had a notch on the recovering phase (Fig. 2A) and sometimes followed by a slow positive deflection (Fig. 2B). The waveforms of this type of pulse were quite variable and on rare occasions biphasic pulses or almost only a negative phase, were observed. Durations ranged from 5 to 10 msec. Amplitudes were usually 0.3 to 1.5 mV but became greatly reduced as the recording electrode was shifted away from the region immediately adjacent to the nerve ring towards the velum or the subumbrellar region. The same reduction in amplitude also occurred when the electrode was positioned on the inner nerve ring.

An attempt was made to isolate the nerve ring together with the velum from the margin of the umbrella, leaving the ring canal and the sphincter muscle on the bell side. Though clear-cut separation was practically impossible, large spontaneous pulses of the nMP type could be recorded along the base of the isolated velum, probably the outer nerve ring (Fig. 2C). The responses, however, became considerably smaller when the electrode was shifted away about 0.5 mm towards the velum (Fig. 2D). It appears likely, therefore, that nMP's are restricted to the velar edge, possibly to the outer nerve ring.

The nMP's appeared in sequence with a short time lag when they were recorded simultaneously at two sites on the nerve ring (Fig. 2E). When an incision was made through the nerve ring between the two electrodes using a fine razor tip, the coordination of events on the two sides of the cut either persisted or was only temporarily abolished. In either case, the time lag between the response peaks at the two points became markedly longer (Fig. 2F), indicating that coordination can be achieved by some kind of alternative route which exists in surrounding tissues. A further cut across the subtentacular region up to the level of the ocelli resulted in complete abolition of coordinated events even though the velum and the subumbrellar muscle sheet are almost intact. The alternative conduction pathway must therefore lie in the subtentacular region and not in the subumbrellar or velar regions. However, since the nerve rings cannot be cut without damaging adjacent tissues to some extent, the possibility cannot be excluded that some conduction occurs through portions of the subumbrellar and velum lying immediately beside the nerve ring.

Conduction velocities at temperatures of 16–20° C were measured by estimating the delay between the response peaks recorded on the two electrodes following electrical stimulation on the nerve ring. In each preparation they were measured 3–7 times and the values were averaged. The mean value of averages obtained from 6 animals was 70.8 cm/sec (variation, 59.4–81.4 cm/sec).

PSP's were small biphasic events with amplitudes of less than 0.5 mV and durations of 3–5 msec (Fig. 3A). When recordings were done on the outer nerve ring, these pulses were always followed by a slow negative deflection (Fig. 3B). Additionally, the negative deflections were frequently followed by a complex pattern of pulses (Fig. 3C) associated with muscle contraction. Thus it appears that the small biphasic events trigger the swimming muscular movements indirectly through the slow negative deflection. Here, it must be noted that the waveform and the polarity of both the negative deflection and the muscle contraction pulse were greatly changed when recordings were made on the inner nerve ring (Fig. 3D). These points will be described later.

PSP's were not restricted to the outer nerve ring as was the case for nMP's but were recordable on the inner nerve ring without appreciable change in amplitude. However, shifting the electrode away from the nerve ring resulted in an abrupt decrease in amplitude, suggesting that PSP's were generated in the two nerve rings, the inner and the outer.

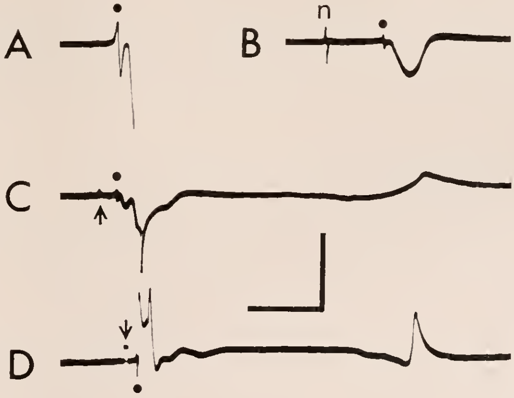

FIGURE 3. Pulses (PSP's) related to trigger muscle contractions. PSP's are indicated by dots and the nMP, by "n." A and B are PSP's shown by fast sweep speeds and C and D show contraction pulses after the PSP's. Note that in B the PSP is followed by a slow negative deflection. C is a record obtained on the outer nerve ring and D, on the inner nerve ring. Vertical bar shows: 0.8 mV in A and 2 mV in B-D. Horizontal bar shows: 23 msec in A; 50 msec in B; 100 msec in C and D. Artifacts by electrical stimulation are indicated by arrows.

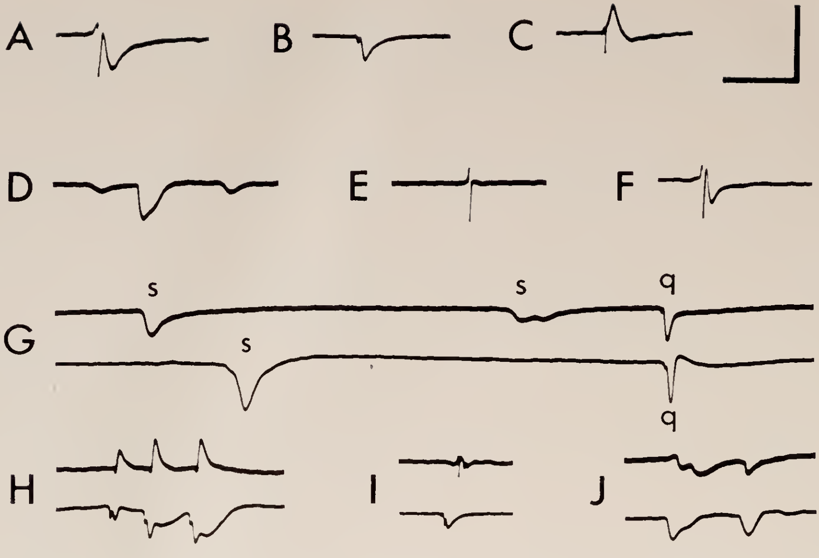

FIGURE 4. Pulses recorded from the subtentacular region and tentacles. A-E are records obtained from the subtentacular region and F, from the tentacle. A and F are records obtained by fast sweep speeds to show waveforms of QSCP's. G shows simultaneous recordings obtained from two radial streaks separated by several radial streaks (s, SMP's; q, QSCP's). In H, the upper trace is a record obtained from the tentacle and the lower trace, from the subtentacular region. I shows simultaneous recordings obtained from two tentacles separated by several radial streaks. J shows simultaneous recordings obtained at two sites along the same radial streak. Vertical bar shows: 2 mV in B, D, H and the upper traces of G and J; 0.8 mV in A, C, E, F, I and the lower trace of G; 1.6 mV in the lower trace of J. Horizontal bar shows: 46 msec in A and F; 100 msec in the others.

Conduction velocities along the nerve ring were measured under the same condition as nMP's. The mean value obtained from 5 animals was 125 cm/sec (variation, 115–142 cm/sec).

Pulses from the subtentacular region and tentacles

Two kinds of pulses were recorded in the subtentacular region. One of them was a compound event consisting of a quick pulse followed by a slower component usually negative (Fig. 4A, B) but occasionally became positive (C). The second type of pulse was a slow negative monophasic pulse (D). Both of these also occurred spontaneously on the nerve ring-free preparations. For convenience sake, the former will be called a quick and slow compound pulse (QSCP) and the latter, a slow monophasic pulse (SMP). In QSCP's, the ratio of the amplitudes of the quick and slow phases was variable, as in Figure 4A and B, and on rare occasions the slow phase was hardly detectable (E).

Since the tentacles are continuous with the radial streaks (Fig. 1C), pulses recorded from tentacles would be expected to show similarities to those recorded from the subtentacular region. Indeed, not only QSCP's (F) but also the SMP's were recordable from the tentacles.

QSCP's appear to be conductive events because simultaneous recordings at two sites in the subtentacular region showed the synchronous or nearly synchronous appearance (Fig. 4G, q). The same was true in preparations of the subtentacular region separated from the nerve ring. Similarly, coordinated QSCP's were obtained in recordings from a radial streak of one tentacle and the tentacle associated with another radial streak several millimeters away (Fig. 4H). Further, coordinated firings were also recorded from two tentacles separated by several radial streaks in the nerve ring-free preparation (Fig. 4I).

On the other hand, SMP's seem to be conducted for a short distance only. As shown in Figure 4G three SMP's (s) occurred independently when the recording sites were separated by several radial streaks. This tendency for independent firings was also detected between adjacent radial streaks. On the same radial streak, coordination, though weak, could be achieved with a slight time lag (Fig. 4J). Conduction velocities could not be measured owing to the short distance involved and the irregular pulse form. Thus SMP's appear to be conducted locally within each radial streak but not across the radial streaks.

The interrelationship between nMP's and QSCP's is important. Though the former usually preceded the latter (Fig. 5A), this does not mean that QSCP's are exclusively efferent events. Indeed as shown in Figure 5B, spontaneous QSCP's preceding nMP's were sometimes observed. The reciprocal elicitation of nMP's and QSCP's implies that the nerve ring, the subtentacular region and the tentacles form one through conducting system connected mutually all over the area and that the relay circuit whose existence is required to explain the continued coordination of nMP's following incision of the nerve rings between two recording sites (see above) is probably achieved by the QSCP's in the subtentacular region.

The non-polarized conduction of the QSCP's can be unequivocally demonstrated by recordings made from animals with intact tentacles. Two recording electrodes were placed about 1 cm apart on the same tentacle and electrical stimuli were delivered either to a place more distal to the recording sites on the same tentacle (Fig. 5C) or to a tentacle separated by 6 radial streaks from the recording sites (Fig. 5D). In Figure 5C, the sequence of events was from distal (lower record) to proximal (upper), whereas in Figure 5D, the sequence was reversed, *i.e.,* from

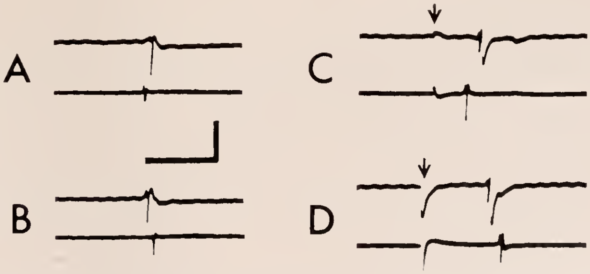

FIGURE 5. Non-polarized conduction of QSCP's and their relation to nMP's. In A and B, the upper traces show spontaneous pulses obtained from the subtentacular region showing QSCP's and the lower traces, from the outer nerve ring showing nMP's. C and D are records obtained by electrical stimulation (arrows) from the tentacles showing QSCP's. Vertical bar shows: 1 mV in C, D and the upper traces of A and B; 2 mV in the lower traces of A and B. Horizontal bar shows 100 msec.

proximal (upper) to distal (lower). The conduction of QSCP's must therefore be non-polarized. In both cases, the time lag between the two pulses was 16 msec so that a rough estimation of the conduction velocity of the QSCP's along the tentacle would be 63 cm/sec. It follows from these observations that excitation can travel up a tentacle proximally in the form of QSCP's, reach the radial streak, and then propagate circularly towards other tentacles via the subtentacular region and pass distally to the tentacle. The latencies after stimulus in Figure 5D being larger than in Figure 5C can be explained by the longer distance for the pulses to travel.

Contraction pulses of the velum and the subumbrellar muscle sheet

When the recording electrode was placed on the subumbrellar side of the velum, two successive slow potential changes separated by a steady phase were recorded (Fig. 6A, B). Their amplitudes ranged from 1–4 mV and the durations measured from the beginning of the first phase to the end of the second one were 250–500 msec. The coupled occurrence of the two potential changes was also observed on the subumbrellar muscle sheet (Fig. 6C, D; amplitudes, 0.5–2 mV; durations, 200–500 msec) but here the waveforms were somewhat variable, the first phases sometimes taking a multiple form. The two types of pulses from the velum and the subumbrella resembled each other closely in waveform (Fig. 6, A to C and B to D). They always occurred in association with swimming movements of the respective muscle tissues. We therefore call them the velar swim pulse (VSP) and the subumbrellar swim pulse (SSP's). The complicated waveform following the PSP shown in Figure 3D probably represents a composite event of the two types of swim pulses.

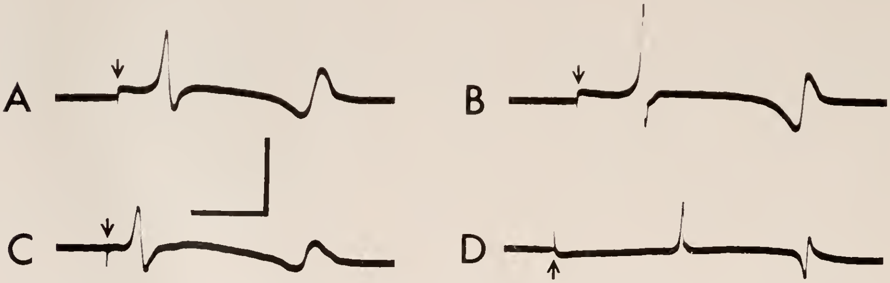

FIGURE 6. The contraction pulses of the velar and the subumbrellar muscles. A and B are records obtained on the subumbrellar-side surface of the velum and C and D, on the subumbrellar muscle sheet. Arrows indicate artifacts by electrical stimulation. All recordings were done with the floating electrode. Vertical bar shows 2 mV. Horizontal bar shows 100 msec.

It is interesting that conduction over the surface of the subumbrella was much faster circularly than radially. Conduction velocities of SSP's were estimated from time differences using the peaks of the first phases as recorded at two electrodes following stimulation. The values as obtained from 5 animals at 16–21° C were found to vary from 13.4–24.5 cm/sec (18.2 cm/sec on average) for circular conduction and from 4.8–8.8 cm/sec (6.6 cm/sec on average) for radial conduction.

The polarity of the first phase depends on the site of the active electrode with respect to the indifferent one (Fig. 7). Here, the velum was dissected away from the nerve ring and the active electrode was placed either on the subumbrellar (A, left) or the exumbrellar (A, right) side of the same isolated velum, placed on the indifferent electrode. The first phase always appeared in opposite polarities in such recordings while the polarity of the second phase was usually unaltered.

Electrical activities over the subumbrellar surface were studied in more detail. According to Mackie and Passano (1968) the umbrellas of *Euphysa* and *Sarsia* consist of 5 layers, *i.e.*, the subumbrellar ectoderm, mesogloea, endodermal lamella, again mesogloea and exumbrellar ectoderm. In *Spirocodon*, the umbrellar tissues could also be separated into five layers under the polarized dissecting microscope. The first, consisting of thin regular and fibrous components, could be torn off by means of a fine forceps in the circular direction, which is the direction in which the muscle is orientated. This layer is probably the subumbrellar ectoderm. The second, probably the mesogloea, was also thin but not directional. Beneath it there was a slightly opaque and very thin layer, presumably the endoderm. The presumed endoderm could not be exposed uninjured. On peeling this layer off, the transparent and thick mesogloea again appeared. The mesogloea was covered by the exumbrellar ectoderm which was vulnerable to and difficult to isolate.

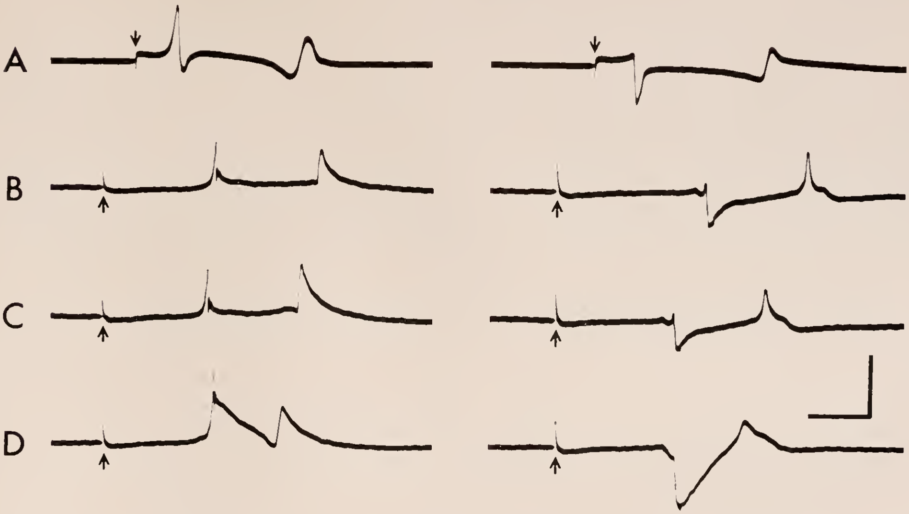

FIGURE 7. Polarity reversal of VSP's and SSP's. A shows VSP's recorded on the subumbrellar side (left) and on the exumbrellar side (right) of the velum. B-D show SSP's obtained by successive electrical stimuli from the subumbrellar muscle sheet (left) and from the mesogloeal side (right). Stimulus artifacts are shown by arrows. Vertical bar shows: 2 mV in A and 0.8 mV in B-D. Horizontal bar shows 100 msec.

In the following experiments, a part of the subumbrellar muscle sheet and the thin mesogloea supporting it were isolated from the umbrella by means of a fine razor. The responses in Figure 7B, C and D were evoked by three successive stimuli (B to D) delivered at intervals of 0.9–1.3 sec. The left column shows records with the subumbrellar muscle side up, the mesogloea side being applied to the indifferent electrode. The SSP's showed waveforms similar to those of intact tissues. The increased positivity of the first phase both in amplitude and in the falling phase which appeared in response to the 3rd stimulus (D, left) was often observed after several repetitive stimuli. The recordings of the right column were done after the same preparation was turned over, and the electode was repositioned in the site corresponding to that in the previous recordings. It is noteworthy that the first phase, being positive on the left, became negative on the right and that in D, the increased positivity of the first phase on the left reflected as the increase in negativity on the right. On the other hand, the polarity of the second phase, being only positive in this case, did not alter in spite of the changes in the waveforms.

Pulses on the exumbrellar ectoderm and the endodermal lamella

It has been described in some hydromedusae that the exumbrellar ectoderm and the subumbrellar endoderm are completely nerve free, but that conductive pulses are recordable from them (Mackie and Passano, 1968). Figure 8A and B shows pulses evoked on the exumbrella by electrical stimuli. They were usually diphasic with amplitudes of 0.4–1.0 mV (A), and sometimes showed a notch in the course of the deflection (B), suggesting the existence of multiple components. Conduction velocities were measured at 16–21° C, using 6 animals and 2 measure-

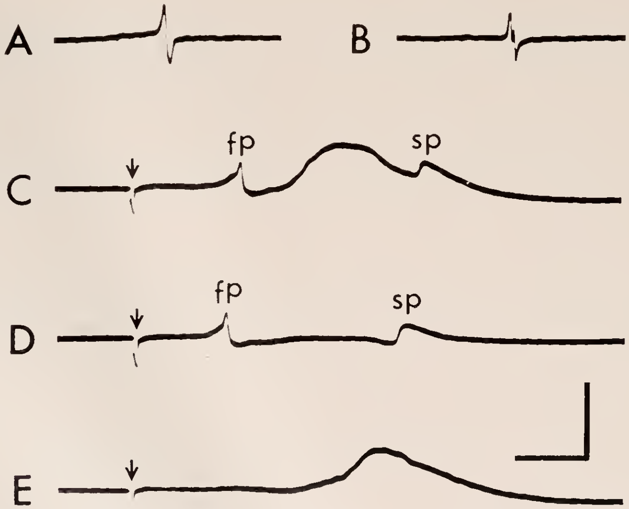

FIGURE 8. Epithelial pulses (EP's) and endodermal pulses (EDP's). A and B show EP's recorded on the exumbrella. C and D are records obtained by successive stimuli on the intact subumbrellar muscle sheet, showing SSP's whose first and second phases are indicated by fp's and sp's. The EDP in between the two phases in C disappeared in D. E shows the EDP recorded on the mesogloea after the subumbrellar muscle sheet was peeled off. Vertical bar shows: 0.8 mV in A and B; 2 mV in C-E. Horizontal bar shows 100 msec.

ments on each. The mean value was 12.2 cm/sec (variation, 7.8–14.3 cm/sec). Their waveforms were like those of nMP's to some extent but it was easy to distinguish them from nMP's by virtue of the much longer durations (15–30 msec) and the slower conduction velocities. They are possibly the epithelial pulses widely observed in hydromedusae and will be abbreviated as "EP". The relation between the EP's and all the other pulses still remains uncertain.

Slow deflections, monophasic or with multiple peaks, could be recorded on the subumbrellar muscle sheet. They were often accompanied by SSP's (Fig. 8C), but when repetitive stimuli at 1 cycle/sec were delivered to the subumbrella, this new type of pulses disappeared after one or two responses, leaving only the SSP's (Fig. 8D). On the other hand, removal of the subumbrellar muscle sheet resulted in complete abolition of the SSP as expected but the slow monophasic deflections still remained (Fig. 8E). Stripping off the mesogloea on the subumbrellar side, which was destructive for the endodermal cells, caused disappearance of this pulse. It follows then that the present type of pulse is not originated in the subumbrellar muscle but in the endodermal lamella. It will be called an endodermal pulse (EDP).

Electrical activities induced by shading

As described above, *Spirocodon* responds to shading by initiating swimming pulsations. The shadow reflex must involve a central coordinating pathway such as the nerve ring. Indeed, in response to light-off the pulses except for EP's and EDP's appeared singly or in combination. An example is shown in Figure 9A in which the PSP, the VSP and the nMP appeared in sequence on the outer nerve ring. The latencies measured from the light-off to the first pulse ranged between 0.2 and 1.1 sec irrespective of the pulse types. Responses were never observed at "on" of a strong light after a period of dark adaptation, however long.

When a cut was made in parallel with a line of the ocelli across the radial streaks, responses were completely abolished on the other side of the ocelli. On the ocellar side, however, both QSCP's and SMP's were induced in response to light-off (Fig. 9B). Further, as shown in Figure 9C the QSCP recorded on a tentacle was undoubtedly conducted from the proximal side (lower trace) to the distal end (upper trace). It appears as if the QSCP was information carrier of light-off generated in the ocelli. However, though QSCP's and SMP's are evoked by light-off without the nerve ring, they cannot be assumed to be the information carrier because upon shading QSCP's usually appeared after nMP's and appearance of SMP's does not coordinate with pulses in the nerve ring (Fig. 9D, E). There must be an event or events which carry information of shading from ocelli through the subtentacular region towards the nerve ring and trigger nMP's, PSP's and indirectly muscle contraction pulses (SSP's, VSP's). At present, we have not observed such an electrical correlate to carry information of shading.

DISCUSSION

Nervous activities in hydrozoa have been studied mainly from the behavioral point of view (for review, see Bullock, 1965) and it is ironic that an important outcome of the recent work is the finding of non-nervous, or neuroid conduction.

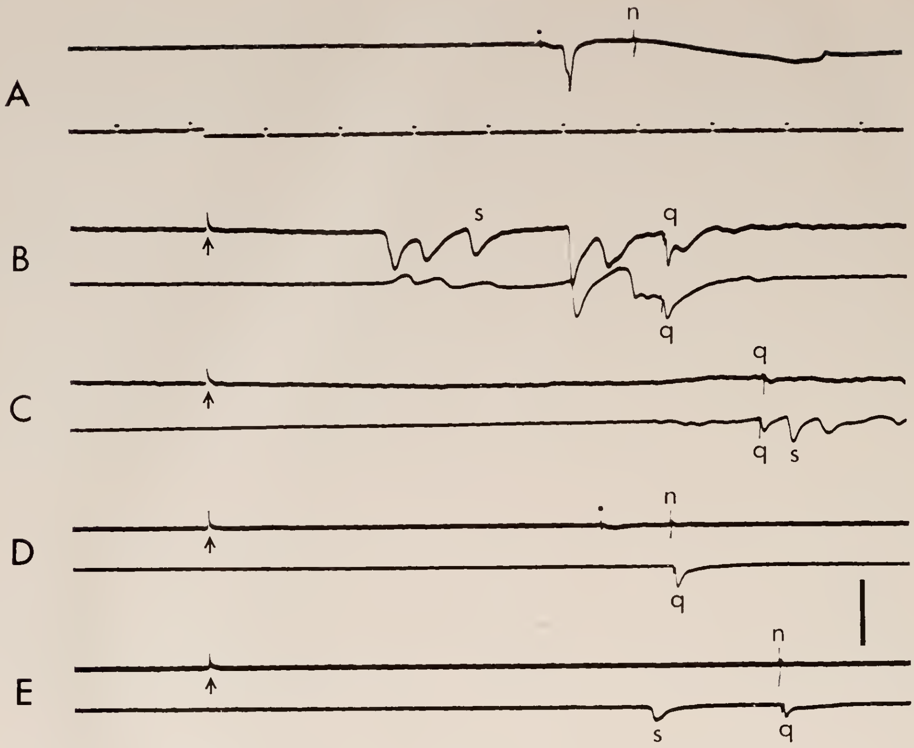

FIGURE 9. Light-off responses of various types of pulses. The upper trace of A is a record from the outer nerve ring. In B, the upper trace is a record from the tentacular base and the lower trace, from the subtentacular region. In C, the upper trace is a record from the distal side of the tentacle and lower trace, from the proximal side of the same tentacle. Experiments in B and C were done on nerve ring-free preparations. In D and E, the upper traces are records on the outer nerve ring and the lower ones, from the subtentacular region. The downward shift of the lower trace of A shows light-off. Arrows indicate stimulus artifacts by light-off. The time mark in the lower trace of A shows 100 msec. B-E were swept at the same rate as A. PSP's are indicated by dots, nMP's by "n," QSCP's by "q" and SMP's by "s." Vertical bar shows: 2 mV in A, D and E; 0.8 mV in B and C.

In his review, Mackie (1970) is of opinion that pacemaker function is restricted to the nervous system in hydromedusae. Since Romanes (1876), rhythmic swimming pulsations in hydromedusae have been known to be under the control of pacemakers around the bell margin, but direct evidence of nerve impulses is lacking.

As mentioned above, we have recorded in *Spirocodon* two types of pulses (nMP's and PSP's) from around the nerve ring. One should be very cautious in assuming pulses taken from hydrozoans to be nervous in origin, for there is ample evidence that non-nervous epithelial pulses do occur (Mackie, 1965; Mackie and Passano, 1968; Josephson and Macklin, 1967). They are characterized by their long time course, slow conduction velocity and extensive distribution, while nervous events seem to be recorded only in the region of the nerve ring. Considering the restricted occurrence to the nerve ring and also the shorter duration and the faster conduction velocity, nMP's and PSP's are best interpreted as reflections of nervous activities. The quick conduction of those pulses would be understandable if giant fibers, as demonstrated in the inner and the outer nerve rings of *Sarsia* (Mackie, 1971), are also present in *Spirocodon*.

As mentioned earlier, nMP's are considered to originate in the outer nerve ring, because of the reduction in amplitude when they are recorded from the inner nerve ring. The PSP's, on the other hand, trigger muscle activities (VSP's and SSP's), suggesting that they reflect activities of motoneurones. If the inner nerve ring consists, as suggested by Bullock (1965), mainly of motoneurones and interneurones, PSP's may be assumed to originate in the inner ring. Indeed, in contrast to nMP's, PSP's can be recorded without reduction in size on the inner nerve ring. The fact that PSP's occur on the outer nerve ring might be explained by reference to the observation that in several hydromedusae nerve fibers run from the inner nerve ring into the outer one across the mesogloea.

The durations of those pulses which have hitherto been assumed to be of nervous origin in hydrozoans are longer than 10 msec. In this respect, PSP's (3–5) msec) and nMP's (5–10 msec) are unusually short but not out of the range of those obtained from other classes of Cnidaria. Indeed, the pulse duration of the single nerve fiber of *Aurelia aurita* has been reported to be less than 0.6 msec (Bergström, 1971). However, it appears unlikely that nMP's picked up by metal electrodes reflect activities of single nerve fibers, because it is difficult to conceive that in the nerve ring which consists of a large number of nerve fibers, only one is active. Instead, we assume that the pulses picked up are mass potentials.

QSCP's are composed of two phases, the first quick one seemingly triggering the second slow one in its passage. The quick phase not only follows upon but also precedes nMP's. The conduction velocities of the two are roughly the same and therefore it may be inferred that the first phase is of nervous origin. The extensive distribution of the QSCP's as well as the direction of conduction, which can be either efferent or afferent, is not surprising, since diffuse nerve nets are widely known to occur within various epithelia in hydromedusae (Mackie and Passano, 1968). This type of pulse is most likely responsible for transmitting information between the periphery and the center, or between two remote sites in periphery. In this respect, it is rather difficult to understand why the QSCP's cannot play an afferent role for shadow signals.

It is significant that the polarity of the first phase of VSP's becomes reversed by turning over the preparation. This observation can be well explained by adopting the idea of an electrogenic layer which was first proposed for interpreting the polarity reversal of ERG by Tomita (1950). The electrogenic layer must lie in parallel with the surface of the velum, the subumbrellar side becoming more positive with respect to the exumbrellar side when excited. The recording from the subumbrellar side of the velum with the indifferent electrode along the exumbrellar side will result in the positive first phase and when reversed, the negative. When myoepithelial cells are excited a current flow might occur from the epithelial surface to the muscular bases of the myoepithelial cells, or alternatively a steady current which flows towards the epithelial surface in the resting state might decrease. What might make such a current flow is not clear, but it may be suggested that the structural inhomogeneity of myoepithelial cells could help to develop a sink and source relationship in the cross-sectional direction. On the other hand, it is considered that the second phase whose polarity remains unaltered is evoked in a way common to the usual action potentials. The bundles of basal muscular processes might be responsible for generating the second component. The above arguments would also apply in the case of SSP's.

Spirocodon responded to light-off by firing various types of pulses and from the experiments shown in Figure 9, it is considered that the information of light-off originates first in the ocelli and is conducted to the nerve ring. Though the pulses which carry the original signal from the ocelli have not been recorded as yet, many pulses recorded in response to light-off give a clue to study information pathways from the photoreceptive sites to the center and hence to effectors such as the tentacles and locomotory apparatus.

We should like to thank Professor G. O. Mackie of Victoria University, Canada for his invaluable help in preparing manuscript and also Professor M. Tamasige of Hokkaido University, Japan for his suggestion to use the polarized light for inspection.

SUMMARY

1. Electrical activities occurring spontaneously and in response to electrical or photic stimulation were recorded from nervous, muscular and non-nervous tissues of *Spirocodon saltatrix*.

2. Two types of quick pulses, nMP's and PSP's, were recorded from the nerve ring. nMP's originated in the outer nerve ring and PSP's, in both the outer and the inner nerve rings. The PSP's appeared to trigger swimming contractions because they always preceded the muscle contraction pulses of the bell (SSP's) and the velum (VSP's).

3. Composite pulses with quick and slow phases (QSCP's) and slow monophasic pulses (SMP's) were recorded on the subtentacular region and the tentacles, respectively. The QSCP's had an intimate relationship with nMP's and their quick components appeared to be of nervous origin as were nMP's and PSP's. The SMP's seemed to be myonal or epithelial events and their conduction was restricted to each radial streak.

4. Contraction pulses of the velum (VSP's) and the subumbrellar muscle (SSP's) consisted of two phases and a steady phase between the two. The electrogenic site of the first phase was discussed from the results of polarity reversal when the positions of the recording and the indifferent electrodes were reversed.

5. Pulses (EDP's and EP's) also occurred in response to electrical stimulation on the exumbrellar surface and the subumbrellar endodermal lamella, respectively.

6. All types of pulses except the EDP's and EP's occurred upon light-off but none of them responded to light-on. Though the information of light-off was considered to originate in each ocellus, no electrical correlate as regards the information carrier has been observed.

LITERATURE CITED

Bergström, R. M., 1971. Unit spike activity in the coelenteran neural network. *Naturwissenschaften*, **58**: 153.

Bullock, T. H., 1965. Coelenterates and Ctenophora. Pages 459–534 in T. H. Bullock and G. A. Horridge, Eds., *Structure and Function in the Nervous Systems of Invertebrates.* W. H. Freeman Co., San Francisco.

Eakin, R. M., and J. A. Westfall, 1962. Fine structure of photoreceptors in the hydromedusan, *Polyorchis penicillatus. Proc. Nat. Acad. Sci.,* **48** : 826–833.

Hisada, M., 1956. A study on the photoreceptor of a medusa, *Spirocodon saltatrix. J. Fac. Sci. Hokkaido Univ., Series VI,* **12** : 529–533.

Jha, R. K., and G. O. Mackie, 1967. The recognition, distribution and ultrastructure of hydrozoan nerve elements. *J. Morphol.,* **123** : 43–62.

Josephson, R. K., and M. Macklin, 1967. Transepithelial potentials in hydra. *Science,* **156** : 1629–1631.

Kikuchi, K., 1947. On the shadow reflex of *Spirocodon saltatrix* and their vertical distribution in the sea. *Zool. Mag. Tokyo,* **57** : 144–146 (in Japanese).

Mackie, G. O., 1965. Conduction in the nerve-free epitheila of siphonophores. *Amer. Zool.,* **5** : 439–453.

Mackie, G. O., 1968. Electrical activity in the hydroid *Cordylophora. J. Exp. Biol.,* **49** : 387–400.

Mackie, G. O., 1970. Neuroid conduction and the evolution of conducting tissues. *Quart. Rev. Biol.,* **45** : 319–332.

Mackie, G. O., 1971. Neurological complexity in medusae: A report of central nervous organization in *Sarsia.* Pages 269–280 in *Actas del I Simposio Internacional de Zoofilogenia.* University of Salamanca, Mexico.

Mackie, G. O., and L. M. Passano, 1968. Epithelial conduction in hydromedusae. *J. Gen. Physiol.,* **52** : 600–621.

Morin, J. G., and I. M. Cooke, 1971. Behavioural physiology of the colonial hydroid *Obelia.* III. Characteristics of the bioluminescent system. *J. Exp. Biol.,* **54** : 723–735.

Passano, L. M., and C. B. McCullough, 1962. The light response and the rhythmic potentials of hydra. *Proc. Nat. Acad. Sci.,* **48** : 1376–1382.

Passano, L. M., and C. B. McCullough, 1964. Co-ordinating systems and behaviour in hydra. I. Pacemaker system of the periodic contractions. *J. Exp. Biol.,* **41** : 643–664.

Passano, L. M., and C. B. McCullough, 1965. Co-ordinating systems and behaviour in hydra. II. The rhythmic potential system. *J. Exp. Biol.,* **42** : 205–231.

Passano, L. M., G. O. Mackie and M. Pavans de Ceccatty, 1967. Physiologie du comportement de l'Hydroméduse *Sarsia tubulosa* Sars. Les systèmes des activités spontanées. *C. R. Acad. Sci. (Paris),* **264** : 614–617.

Romanes, G. J., 1876. Preliminary observations on the locomotor system of medusae. *Phil. Trans. Roy. Soc. London,* **166** : 269–313.

Rushforth, N. B., and D. S. Burke, 1971. Behavioral and electrophysiological studies of hydra. II. Pacemaker activity of isolated tentacles. *Biol. Bull.,* **140** : 502–519.

Tomita, T., 1950. Studies on the intraretinal action potential. I. Relation between the localization of micro-pipette in the retina and the shape of the intraretinal action potential. *Jap. J. Physiol.,* **1** : 110–117.

Uchida, T., 1927. Studies on Japanese hydromedusae. I. Anthomedusae. *J. Fac. Sci. Imp. Univ. Tokyo, Section IV,* **1** : 145–241.

Yoshida, M., 1959. Spawning in coelenterates. *Experientia,* **15** : 11–13.

Yoshida, M., 1969. The ocellar pigment of the anthomedusa, *Spirocodon saltatrix;* Does its photoreduction bear any physiological significance? *Bull. Mar. Biol. St. Asamushi,* **13** : 215–219.

Yoshida, M., H. Ohtsuki and S. Suguri, 1967. Ommochrome from anthomedusan ocelli and its photoreduction. *Photochem. Photobiol.,* **6** : 875–884.

Reference: *Biol. Bull.*, **145**: 548–564. (December, 1973)

NATURAL HISTORY OF THE HYDROCORAL *ALLOPORA CALIFORNICA* VERRILL (1866)[1]

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The hydrocoral *Allopora californica* Verrill (1866) is one of many spectacular, but little known organisms living subtidally off the central California coast. Until recently its habitat frustrated attempts at scientific investigations for dense kelp beds and the rocky reefs in the area made dredging difficult. Even when specimens were dredged, it was hard to envision the environment from which they had come. Of course there was no way to revisit the same coral colonies repeatedly, so long-term investigations were not possible. Not until SCUBA (Self Contained Underwater Breathing Apparatus) was developed could this rich environment be opened to direct scientific investigation. Using this research tool, this study of *Allopora californica* was undertaken, the first attempt to observe the living hydrocoral in its normal undisturbed situation underwater. The life history, settlement and mortality of new colonies, and regeneration were studied.

Materials and Methods

SCUBA procedures

All dives were made with a "buddy," thanks to the willing help of many University of California certified divers, and all diving procedures were in accordance with the U. C. Berkeley Diving Safety Manual. The dives were "no-decompression" dives conforming to the U. S. Navy Air Decompression Tables (U. S. Navy Diving Manual, 1963). The study extended from November 1968 through May 1971 except for the period from October 1969 through December 1969 when the Carmel beaches were closed as a result of serious pollution from sewer outfalls.

Research sites

The two research sites were 15–20 meters deep, one off Carmel River Beach, Carmel, California, and the other in Bluefish Cove at Point Lobos State Reserve, Carmel, California (Fig. 1). Each site was marked with a surface float to facilitate relocation. The underwater areas were mapped making it possible to swim to any part of the site, even when underwater visibility was limited to a few meters (Little, 1971).

At each site large, irregularly shaped rocky reefs rise 3 to 5 meters above a sand or rubble bottom. The tops of the reefs are about 15 meters deep, while the sandy bottoms are about 20 meters deep. *Macrocystis integrifolia* is the dominant

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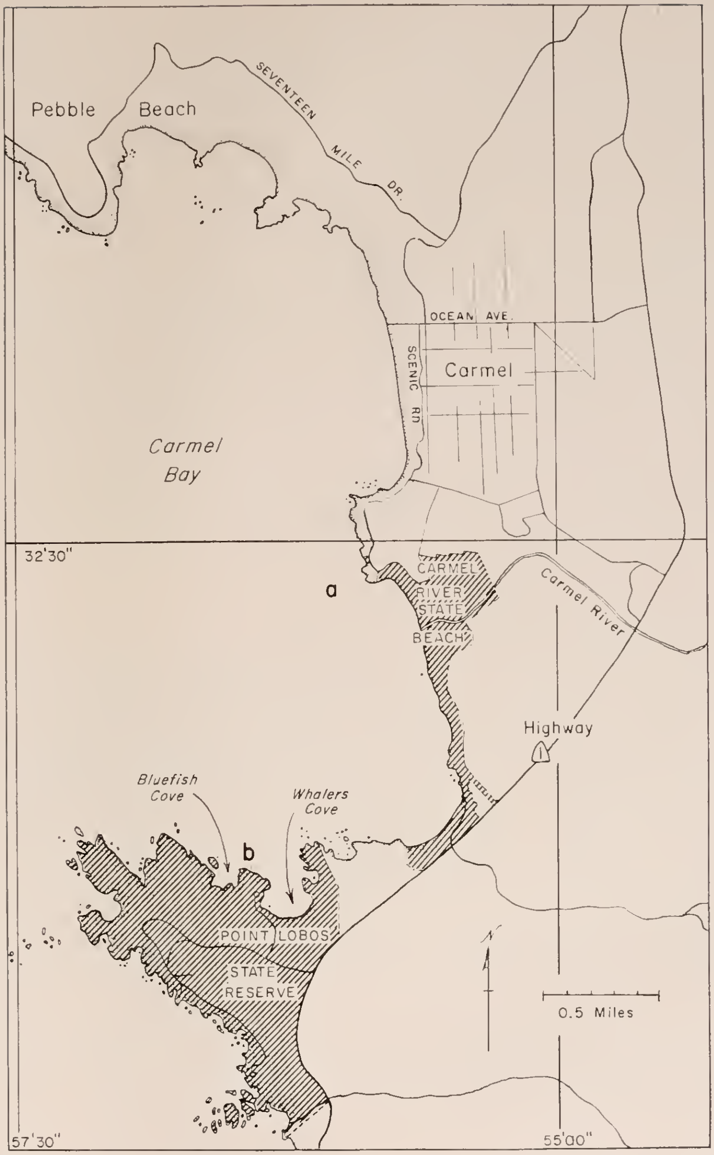
Pebble Beach
SEVENTEEN MILE DR.
OCEAN AVE.
SCENIC RD
Carmel
Carmel Bay
32'30"
a
CARMEL RIVER STATE BEACH
Carmel River
Highway 1
Bluefish Cove
Whalers Cove
b
POINT LOBOS STATE RESERVE
N
0.5 Miles
57'30"
55'00"

FIGURE 1. Map of Carmel Bay, Carmel, California, showing the location of the two research sites; (a) Carmel River Beach research site, (b) Bluefish Cove research site.

alga. Various small red, brown and coralline algae cover the tops of the reefs during certain seasons of the year. The research areas are rich in invertebrate life with many species present. Some of the more delicate sponges, bryozoans, tube worms, *etc.* are common here. Several open-water rock fish, *Sebastes* spp., are seen, as well as many of the bottom-dwelling territorial species.

Life history study

For the study of the reproductive cycle, specimens of coral were collected approximately monthly from both sites. A small branch was collected from each of 20 tagged colonies and from a few randomly selected colonies. As soon as possible after returning to the beach, the samples were relaxed for 1–2 hours in sea water with a few menthol crystals sprinkled on the surface, killed with 1–2% formalin, in sea water (10 minutes), then fixed in Bouin's picroformalin fixative or Susa's mercuric-chloride fixative. The coral was decalicified using daily changes of the fixative. A week to 10 days was usually required.

When the skeleton had been decalcified, a gross examination was made with the aid of a Wild dissecting microscope. The size of the piece was determined, the number of cyclosystems counted, and then, if the sample were female, the tissue was teased apart to pick out the eggs. These were counted and measured to obtain an average size. If it were a male colony, the general condition of the sperm sacs was recorded. A second piece of the same branch was then processed for paraffin sectioning using cedarwood oil as a clearing agent to help soften the yolky tissue. Sections were cut at 8 μ, stained with Heidenhain's iron hematoxylin, and occasionally counter-stained with eosin Y or fast green. From these slides the state of the development of the reproductive products was determined.

Settlement and mortality of new colonies

In order to study larval settlement and subsequent survival a recruitment-mortality study was made. A definable rock surface was chosen and a reference piton driven in. A measuring line with distances marked was used to measure the distance from the piton to each colony on the rock. A plastic protractor, fitted with a clip which slipped over the piton, was used to measure direction. For each colony two coordinates were recorded: a distance and a direction in degrees relative to the reference piton. An underwater slate was used to record the data (Little, 1971). When checking the study areas, previously existing colonies were located and the positions of new colonies were added to the slate. An underwater light was indispensible for spotting the tiny new colonies. Eight study plots, four at each research site, were studied for almost two years with counts made every 1–2 months.

The size of these irregular plots was determined by measuring the distance and direction from the reference piton to the edge of the rock at several points. This information was transferred to graph paper and, when the outline was drawn, the area could be estimated.

Regeneration

For the regeneration study, large colonies of *Allopora* were tagged and 5–10 separate branches were snapped off. Close-up photographs of the cut ends (1:1 subject:image ratio) were taken periodically to assess the extent of regeneration. All underwater photographs in the study were taken with a Nikonos 35 mm underwater camera with a Nikonos flash attachment, on Kodachrome X film (ASA 64), using Sylvania 26B or General Electric 6B flashbulbs. For a 1:1 subject:image ratio a commercially available extension tube was used with the 35 mm lens.

In addition to the photographic record made in the field, regeneration was studied histologically. At various intervals cut branches were recut. These were fixed, decalcified, and sectioned in paraffin for light microscopy as described previously. By examining these sections it was possible to trace the healing of the cut surfaces and the development of new cyclosystems as the branches regenerated

RESULTS

General structure

Allopora californica is abundant in parts of Carmel Bay at depths of 10–30 meters. Living colonies may be pink, ranging from a very light pink to a light red, or they may be various shades of purple. The largest are 30 cm high and may be 30 cm in diameter. The growth form is variable, sometimes as regular as a hemisphere in shape, but usually displaying an irregular branching pattern. The rows of branches tend to grow perpendicular to the prevailing currents (Roth, 1969).

On the colony surface the openings, or cyclosystems, are visible. These pores each contain a single gastrozooid, or feeding polyp, plus 4–8 dactylozooids, which are protective and food-capturing polyps. In a longitudinal section through the gastrozooid and dactylozooids, the cyclosystem appears as indicated in Figures 2 and 3.

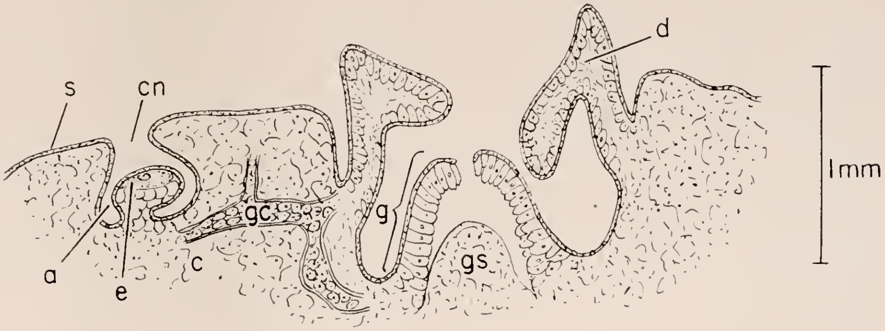

FIGURE 2. Diagram illustrating a typical longitudinal section through a cyclosystem of *A. californica*. Abbreviations used are: a, ampulla; c, calcareous skeleton; cn, canal opening to the surface of the colony; d, dactylozooid; e, egg; g, gastrozooid; gc, gastrodermal canals; gs, gastrostyle; s, surface of the colony.

The gastrozooids are 0.5–1 mm deep in the cyclosystems, each located on a calcareous spine, the gastrostyle. The gastrozooids have four tiny, seemingly useless tentacles around the mouth. The surrounding dactylozooids are also located atop calcareous structures, the dactylostyles. The mouthless dactylozooids can extend and reach 1–2 mm above the surface of the colony to capture food. Connecting the different zooids and cyclosystems within a colony there is a series of tortuous canals composed of gastrodermis with a thin epidermal covering. The mesolamella between the two layers is acellular and extremely thin. Presumably nutrients and waste products can be moved via the gastrodermal canals. The living tissues, the zooids and canals, are found primarily in the outer 2–4 mm of the colony. Calcified material fills the spaces not occupied by the living tissue. The inner core of the colony is calcified material with only a few strands of living tissue running through it.

A second type of dactylozooid is also present. These are not part of a cyclosystem; rather, they are found singly in chambers scattered at random throughout the colony between cyclosystems. The function of these zooids is not known for certain, but they are the first to expand after the colony has been disturbed, suggesting either a sensory or a protective role.

The male and female reproductive structures or gonophores develop in epidermally-lined cavities, the ampullae. These are generally smaller and more numerous in male colonies.

Three obligate commensals have been reported on *Allopora*, a barnacle, a polychaete worm, and a snail. The barnacle, *Balanus nefrens* Zullo (1963), settles on the surface of the colony. The coral grows over the barnacle leaving only the opening at the top exposed. Most colonies have several barnacles on them, forming pyramid-shaped growths along the sides of the branches.

A second commensal is the spionid worm, *Polydora alloporis* Light (1970). The worms burrow longitudinally through the central calcareous core of the branches, secreting tubes lined with calcareous material. Paired openings to the tubes are found scattered over the surface of the coral colony. Almost every colony found was infested with worms, sometimes so many that the skeleton was weakened and more susceptible to breakage.

On rare occasions, a third commensal, the ovulid snail *Pedicularia californica* Newcomb (1868), was found on *Allopora*. The snail's shell always matched the color of the host colony. On repeated visits to the same colony, the snail was always found in exactly the same place, a slight depression on the surface of a branch exactly the right size and shape to accommodate the margin of the snail's shell. No living cyclosystems were found in this area. Either *Pedicularia* does not move or it always returns to the same position on the branch of coral.

Reproductive cycles

Each colony of *Allopora californica* is either male or female. This cannot be determined in the field; the colony must be dissected or sectioned to determine its sex.

From the histological data the development of the female gonophore, the egg, and the planula was observed. The eggs grow, are fertilized, and develop in the ampullae. These cavities originally form at the lateral margins of the colony. Epidermis and gastrodermis meet, and the cell layers thicken. The branch continues to increase in diameter while the area around the thickening remains fixed, giving the impression that the young ampulla is sinking into the calcareous skeleton. At the stage shown in Figure 4 the tiny ampulla, still open to the outside, is an epidermally lined cavity. The structure in the center is composed of gastrodermal cells with a thin covering of epidermis.

The oögonia arise in the gastrodermal canals. Their precise site of origin is not known, but the distinctive cells can be seen inside the canals (Fig. 5). Meiotic divisions were not observed, so it was not possible to distinguish oögonia from primary and secondary oöcytes. The eggs migrate along the gastrodermal canals to the developing ampullae, and one egg settles between the epidermis and gastrodermis (Fig. 5). Often other eggs are seen close to the ampullae or even at the base of the gastrodermal supporting structure, but only one settles at the position shown. The others probably degenerate. This stage of development, with the tiny egg between the gastrodermis and epidermis, is seen at all times of the year. Apparently more than a year can elapse before the egg begins to grow.

In late December and January the gastrodermal supporting structure, called the trophodisc, begins to increase in size and a distinct lumen becomes visible. Tiny yolk granules appear in the cytoplasm of the egg. The ampulla must increase in size to accommodate the growing egg and trophodisc. This is probably accomplished by dissolution of the surrounding calcareous skeleton. The nucleus of the egg is very distinct, measuring some 30 μ in diameter, and the tiny pore to the outside of the ampulla, diameter 16 μ, is still open.

Through February, March and April the trophodisc further increases in size (Fig. 6). The gastrodermal structure repeatedly outpockets, giving the appearance of numerous blind tunnels. More yolk is deposited, seeming to coalesce to form much larger granules. The largest granules are proximal, while the smaller ones are found at the distal side of the egg. The nucleus is still visible in a position near the distal edge of the egg, always very near the pore leading to the outside. The sperm probably enter the ampulla via this opening, the fertilization canal.

The egg remains at this stage until fertilization, which occurs some time in late May, June, or early July. Actual fertilization was never observed, but after an egg has been fertilized the nucleus is no longer visible, and the fertilization canal closes. The trophodisc begins to diminsh in size and completely degenerates within a few weeks.

The first visible sign of cleavage is a layer of cells at the distal edge and along the sides of the yolk. The yolk does not divide. In later stages these undifferentiated cells surround the yolk completely. As development proceeds, these cells differentiate into columnar epithelial cells with clear cytoplasm at the top and dense granules at the base (Fig. 7). These columnar cells appear first at the distal margin of the embryo, then at the sides, and finally along the proximal margin. Numerous small cells, presumably gastrodermal cells, are seen in the yolky area. Their origin and time of migration is not certain. The epidermal cells continue to increase in size for about two months. The mature, oval-shaped planula larva is 0.5–1 mm in length. Sometimes it must bend back on itself to fit in the tiny ampulla. The escape route for the planula develops after the fertilization canal closes. This is a new opening from the ampulla which opens into a nearby cyclosystem between two dactylozooids. In two favorable instances serial sections were obtained of planulae escaping through this canal. Release of the planulae occurs in late October, November, and early December.

The ampulla left by the escaped planula does not go to waste. Even while the planula is developing, a tiny new trophodisc is forming at the bottom of the ampulla as gastrodermis pushes into a thickened epidermal cell layer. The developing planula grows around this structure (Fig. 7). A new egg migrates to the area and is in position before the planula escapes. In this way the ampullae are used repeatedly.

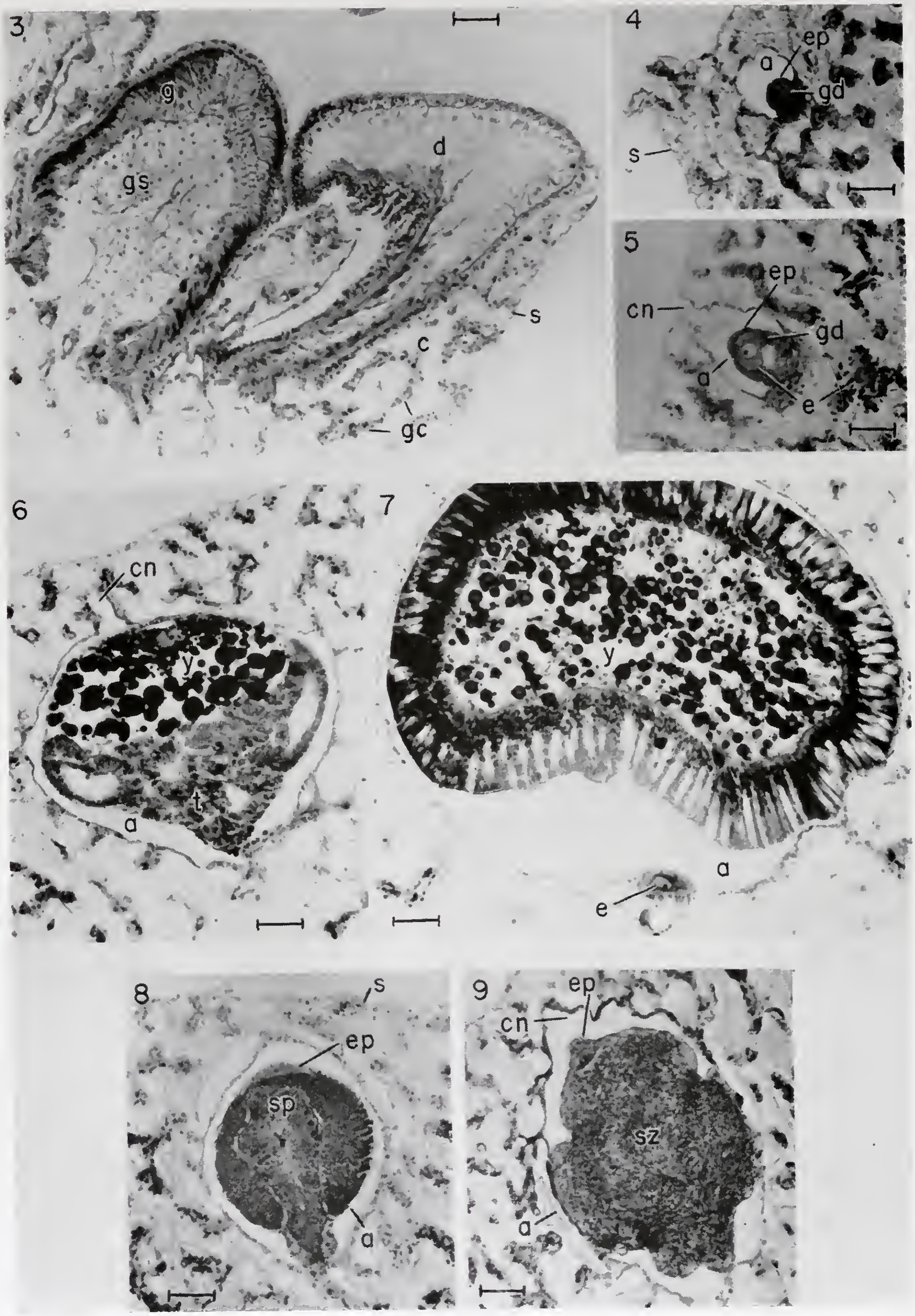
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FIGURE 3. Photomicrograph of an actual longitudinal section through a cyclosystem. Zooids are only partially expanded.

The male gonophores also develop in ampullae. New male ampullae arise in the same way as do female ampullae (Fig. 4). The chamber is usually closed by a lid of tissue in the male ampulla. Spermatogonia arise in the gastrodermal canals and migrate to the developing ampulla, settling between the gastrodermis and epidermis. The cytological events of meiosis were never seen, so spermatogonia, and primary and secondary spermatocytes could not be identified.

Small ampullae with male germ cells are present at all times of the year, indicating, as in the females, that gonophores need not form and mature in a single year. In November and December the male gonophores begin to develop, with the gastrodermis forming a supporting structure, called a spadix, with a large lumen. The cells destined to become sperm cells increase in number, possibly by cell division or by further migration from the gastrodermal canals (Fig. 8). The ampulla is still closed to the outside at this stage.

The gonophores increase in size to about 500 μ. Starting in late February and continuing through April, the cells undergo a change in which the nuclei stain very darkly and are very tightly packed. They remain this way for 4–6 weeks.

Starting in late April the darkly staining cells change into spermatozoa (Fig. 9). In favorable sections sperm sacs containing both darkly staining cells and spermatozoa were seen. The opening to the outside is still barred by a plug formed by layers of epidermal cells at the distal end of the sperm sac. The gastrodermal spadix degenerates, leaving a sac full of sperm.

Spermatozoa are released in June and July. Actual release was seen only once in the histological sections with many sperm free in the ampulla and the sac apparently in the process of decreasing in size. The epidermal plug had opened, allowing the sperm to be released from the sac. Presumably the sperm are carried by currents to nearby female colonies where they enter the ampullae via the fertilization canals to reach the eggs.

Once the sperm are released, the gonophore for the following year begins to form. A new gastrodermal spadix is visible in late summer, usually with only a few germ cells present. No further development is visible until November or December.

The graph in Figure 10 shows the results of gross examination of the colonies. The mean, range, and standard deviation for egg and planula sizes are indicated.

FIGURE 4. Section through the edge of a colony showing a developing ampulla prior to the migration of gametes to the gonophore. Similar development is seen in both male and female colonies.

FIGURE 5. Photomicrograph of an undeveloped egg in the gonophore. Eggs at this stage of development are present throughout the year. Note the egg in the gastrodermal canals.

FIGURE 6. Fully developed female gonophore as typically seen in April and May. The egg nucleus is still visible, close to the proximal end of the fertilization canal.

FIGURE 7. Section through a developing planula showing the distinctive columnar epithelial cells surrounding the yolk. A new gonophore is developing under the planula.

FIGURE 8. Developing male gonophore typical of those seen in January with many future sperm cells in the sac.

FIGURE 9. Photomicrograph of a sperm sac containing spermatozoa. This is typical of male gonophores in May. Abbreviations used in Figures 3 to 9 are: a, ampulla; c, calcareous skeleton; cn, canal opening to the surface of the colony; d, dactylozooid; e, egg; ep, epidermis; g, gastrozooid; gc, gastrodermal canals; gd, gastrodermis; gs, gastrostyle; s, surface of the colony; sp, spadix, sz, spermatozoa; t, trophodisc; y, yolk. Scale equals 50 microns.

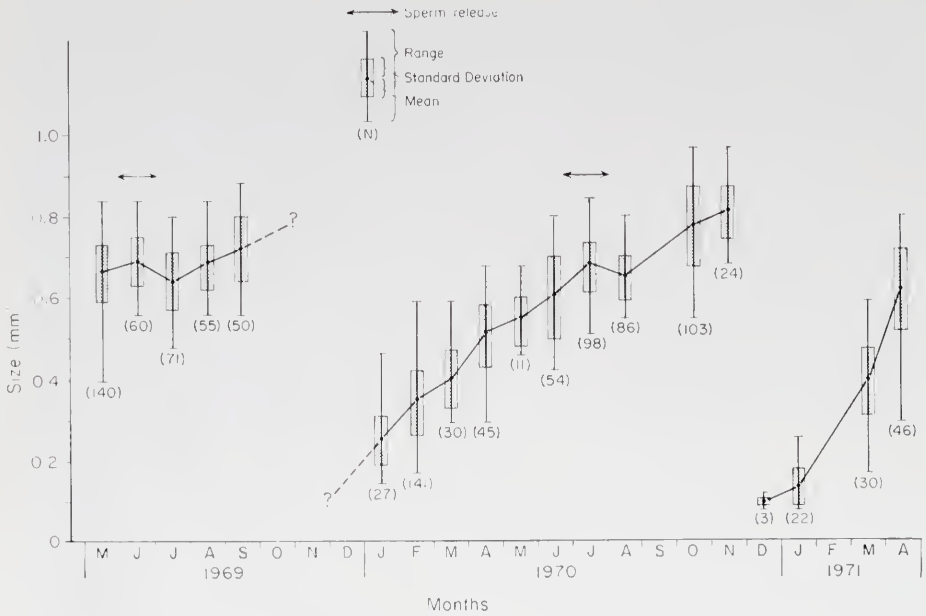


FIGURE 10. This figure shows the average egg or embryo size at different times of the year in *Allopora californica.* See text for further explanation.

There were always eggs present without yolk as indicated earlier (see Fig. 5). These ranged from 40–60 μ in size and could not be seen with the dissecting microscope. Figure 10 represents only those eggs in which yolk deposition had begun, those eggs which would be fertilized and would develop within that year. The double arrow indicates the period of sperm release and, presumably, of fertilization. From this point on, the size measured was that of a developing planula. The break in the graph, October through December 1969, was the period when collections were impossible as the Carmel beaches were closed because of pollution. The probable course of events during late 1969 has been indicated by a broken line.

Within the *Allopora californica* population at the two research sites there was variation in the size of the eggs or planulae at any given time, but there was very little variation in the stage of development. All the eggs began to deposit yolk at about the same time. Essentially all the eggs were fertilized within a 3–4 week period, and development of planulae progressed at a similar rate in all the colonies examined. Most of the planulae escaped in late October and November. A few (3) eggs showed yolk deposition in December 1970, but most showed no sign of growth until January 1971.

Settlement and mortality of new colonies

Eight separate plots totaling almost 4.5 square meters of surface area were studied. When planulae are released, they crawl or are carried by currents away from the parent colony and settle where space permits. A newly settled colony is flat, but even at this early stage the beginning of a depression where the first cyclosystem will form is visible. In four colonies known to be less than three weeks old, the first cyclosystem had already formed.

The maximum possible ages of newly settled colonies were used in constructing a survivorship curve. If an area was examined, say, October 1, November 1, and December 1, and if a new colony was first seen on November 1 but was gone December 1, then the maximum life span was recorded as from October 2 to November 30, or 60 days. Survivorship curves for those colonies which settled during the two years, 1969–1970 and 1970–1971 are shown in Figure 11.

In the curve representing the data for 1969–1970, the solid line indicates actual observations. The dashed portion of the curve, covering the period when direct observations were impossible because of the closing of the beaches, is an estimate assuming that the rate of disappearance was the same for both years of the study. The settlement date was estimated from details of the reproductive cycle and larval development observed through mid-September of that year. Heaviest mortality occurred in the first few months after settlement. At the end of the study, 1½ years later, six colonies were still alive.

The data for 1970–1971 include observations on 128 new colonies. At the end of the study, 180 days later, there were 25 colonies left.

Several factors are responsible for the demise of small colonies of *A. californica*. In many cases they attach to unstable substrates or to short-lived organisms, such as algae or brittle bryozoans. The settling of particulate matter is extremely harmful to the young colonies, and many on horizontal surfaces are buried in this way. Those on vertical surfaces or in areas of heavy surge tend to fare better. Young

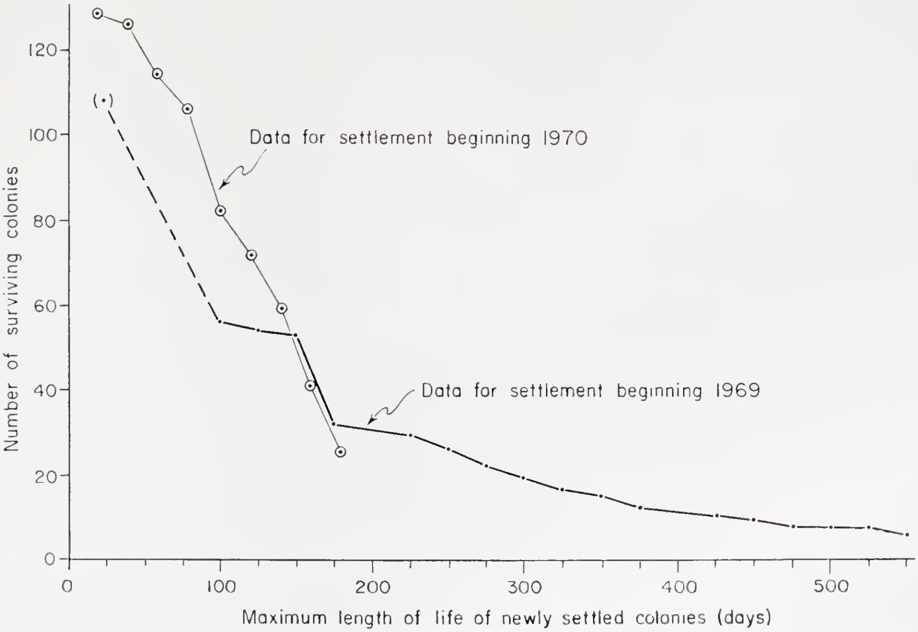
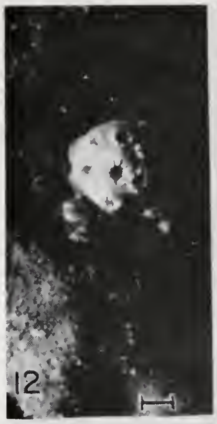
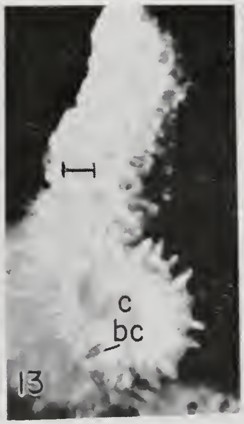
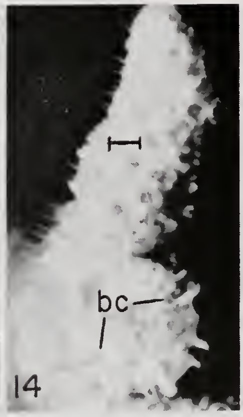
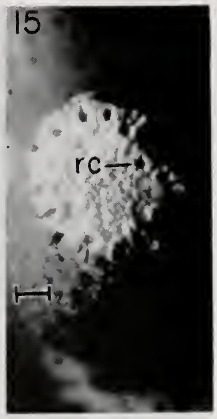


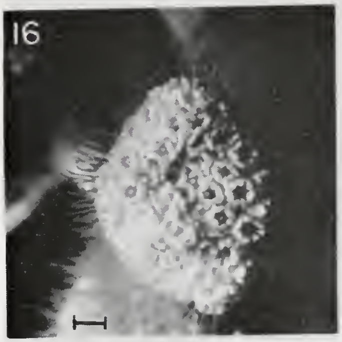
FIGURE 11. This figure shows survivorship curves for *A. californica* colonies settling in 1969 and settling in 1970. Dashed line indicates estimate. See text for further explanation.

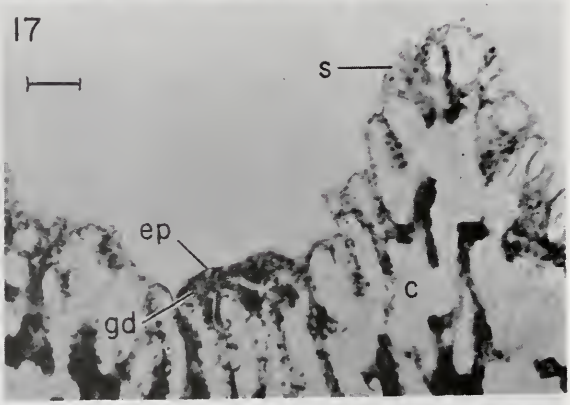


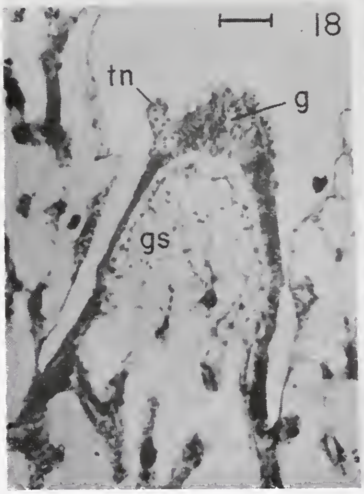












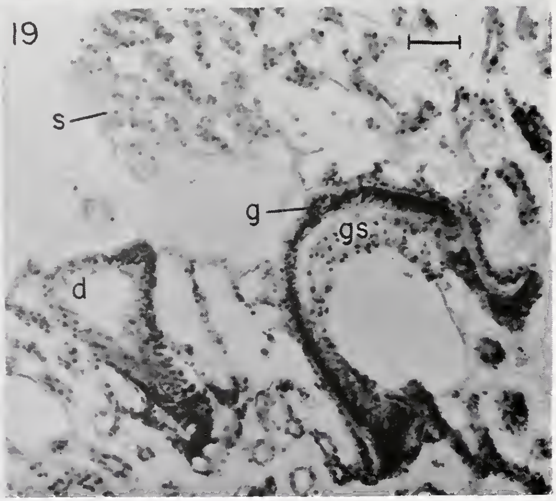


FIGURE 12. In water photograph of a one year old *A. californica* colony with several of its cyclosystems visibile. Note the newly settled barnacles crowding the coral.

colonies must compete with other benthic organisms for settling space. When algae begin to grow rapidly in the early spring, numerous colonies are overgrown. Barnacles and other organisms settling out of the plankton compete with the slow-growing *A. californica*. Figure 12 shows a typical one year old colony, only 5 mm across, with eight cyclosystems.

Regeneration

A newly cut branch of *Allopora* appears as seen in Figure 13. The broken edges are sharp, and the damaged cyclosystems stand out distinctly. The center of the branch is calcareous material. Within 7–14 days a change is evident (Fig. 14). The sharp edges are rounded, and the calcareous core is covered with tissue resembling very fine granulated sugar. This tissue covering continues to grow until it resembles fine sand grains on the tip of the branch. Within 4–6 weeks this granular appearance gives way to a series of ridges and furrows along the broken surface (Fig. 15). The cyclosystems broken in the original cut begin regenerating almost immediately and new cyclosystems are visible as early as 50 days after the cut is made. Broken cyclosystems reorient, if necessary, to resume growth in a direction compatible with the new orientation of the branch. Some reorient in an upward direction toward the new tip of the regenerating branch, while others resume growth outward toward the sides of the branch (Fig. 15). By comparing photographs of the same branch taken over a period of time, the new cyclosystems could be distinguished from those which existed prior to the cut. When new cyclosystems cover most of the available area, upward growth resumes (Fig. 16). This is usually 4–5 months after the branch is broken. The whole tip may grow, or the regenerating area may send up one or more branches with diameters less than that of the original branch.

Periodically the regenerating branches were broken off, fixed, and prepared for histological examination. Within a few days after the original break, the surface is invaded by numerous gastrodermal canals and an epidermal covering layer. The easily-visible granular appearance and ridges result from different

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FIGURE 13. In water photograph of a newly cut branch of *Allopora californica* showing several broken cyclosystems. The central portion of the cut branch is calcareous material.

FIGURE 14. The same branch as seen in Figure 13 photographed 14 days after the branch was cut. The central calcareous core has been covered by living tissue.

FIGURE 15. Regenerating branch photographed on Day 56. The tissue covering the central core appears as ridges and furrows. A regenerating cyclosystem on the right has reoriented to grow upward toward the new surface of the branch.

FIGURE 16. Regenerating branch on Day 235 covered with new cyclosystems. Note the extended dactylozooid tentacles.

FIGURE 17. Photomicrograph of a regenerating branch showing the initial appearance of a new gastrozooid marked by a meeting of epidermis and gastrodermis at the surface of the colony.

FIGURE 18. Section through a new gastrozooid showing further development, including the appearance of one of the minute tentacles on the side of the gastrozooid.

FIGURE 19. Longitudinal section through a complete, tiny, new cyclosystem found on a regenerating branch tip. Abbreviations used in Figures 12 to 19 are: bc, broken cyclosystem; c, calcareous skeleton; d, dactylozooid; ep, epidermis; g, gastrozooid; gd, gastrodermis; gs, gastrostyle; rc, regenerating cyclosystem; s, surface of the colony; tn, tentacle. Scale for Figures 12 to 16 equals 1 millimeter. Scale for Figures 17 to 19 equals 50 microns.

growth rates of the tissue and skeleton resulting in the pitted surface seen in Figure 17. A thickened mass of gastrodermis and epidermis signals the beginning of a new cyclosystem (Fig. 17). The regenerating branch tip continues to grow, leaving this tissue behind in an ever-deepening depression. At a later stage, the future gastrozooid is quite recognizable, complete with a tiny gastrostyle and minute tentacles (Fig. 18). Shortly thereafter, on the walls of the future cyclosystem, the dactylozooids appear. These also start as a meeting of gastrodermal and epidermal tissue layers. The tiny cyclosystem, about $\frac{1}{4}$ full size, is shown in Figure 19.

Discussion

The first work on reproduction in a hydrocoral was by Hickson (1888) on *Millepora plicata.* He described the origin of both male and female gametes in the epidermis lining the gastrodermal canals, including a detailed account of the cytological events of maturation. The male germ cells induce the formation of a male gonophore, usually in the dactylozooids. The fertilized eggs develop in the gastrodermal canals and later at the base of the gastrozooids without any formation of a gonophore. The ciliated larvae without any yolk are released at an early stage. In another species, *Millepora murrayi,* a male gonophore develops, but the eggs develop solely in the gastrodermal canals.

In studying the stylasterine corals, Hickson (1890, 1891) suggested that the gametes cause local irritation in the gastrodermal canals, resulting in the outpocketing of tissue to form gonophores. Gametes in the ampullae are covered by a double sheath of epidermis and gastrodermis and are supported by a trophodisc in the female and a spadix in the male. Sperm enter the female colony via the mouth of the gastrozooids and migrate along the canals to fertilize the egg. Hickson described early planula development, and mentioned the re-use of the female ampullae year after year.

Reproduction in three species of stylasterine corals was described by England (1926). She found that the ampullae form at the surface of the colony where epidermis and gastrodermis meet and form a thickened tissue layer. In contrast to Hickson (1890, 1891), England did not find a double sheath covering the gametes in the gonophore; there was only an epidermal covering and a gastrodermal supporting structure with the egg or sperm between the two layers.

Goedbloed (1962) discussed the origin and development of gonophores in *Allopora blattea* and *Stylaster roseus.* She, too, concluded that the ampullae form at the surface of the colony, and she suggested that the germ cells induce their formation.

The work discussed above was limited to specimens from isolated collections, not allowing repeated observations to determine seasonal changes. The present investigation has been done on material collected monthly over a period of two years, giving a dimension of time to the study.

The ampullae of *Allopora californica* do form at the surface of the colony. With the available histological sequence, the course of events is quite clear; without it, Hickson's suggestion of ampulla formation from the inside out would look quite plausible. No evidence was found to support the assertion that gametes induce the formation of new ampullae. The new ampullae begin without any trace of gametes in the vicinity.

None of the previous workers suggested the dormancy of gonophores such as has been found in this study. Small ampullae with undeveloped future sperm and eggs were found throughout the year (see Fig. 5). All the maturing gonophores in each colony were at about the same stage of development at any one time. There was no indication that a second brood was produced later in the year, hence these immature gonophores must remain in this condition through a whole breeding season. What triggers the beginning or delay of maturation of a gonophore is not known.

Fertilization of the eggs in *A. californica* must be a complex process, since the sperm, released en masse, must travel to the ampullae of distant female colonies. Miller (1966) found evidence of chemotaxis during fertilization of the thecate hydrozoan, *Campanularia.* If a similar mechanism were present in *A. californica,* it would explain how tiny sperm can find the minute fertilization pore. Essentially all eggs examined after the period of sperm release were fertilized; only a very few seemed to be degenerating rather than developing.

The re-use of ampullae year after year is obviously a useful adaptation. Building an ampulla within a calcareous skeleton is a metabolically demanding task. The ampulllae are probably used for several years. As the colony increases in diameter, some are filled in with calcium carbonate and left behind.

A regular yearly cycle of sperm and egg production, fertilization, development, and release of the planulae is clearly demonstrated in *Allopora californica* (see Fig. 10). It is difficult to say what might trigger the cycle. Water temperature is very constant at Carmel, varying haphazardly between 8° C and 12° C at the depth of the study sites. There is rarely any thermocline in Carmel Bay at the depths investigated. Water turbidity is variable, with visibility varying radically even on the same day. Algal growth has a regular cycle with the short algae appearing in March, only to be cut off from the light by the giant kelp, *Macrocystis integrifolia* in June. Which, if any, of these environmental factors influence *Allopora* has not yet been determined. Grigg (1970) found that the reproductive cycle in the gorgonian *Muricea* was different in populations at different depths, possibly correlated with temperature differences. In a very few samples of *Allopora californica* taken from various depths there did not appear to be any difference in development of gonophores or larvae, but a more rigorous study is needed to demonstrate this conclusively.

Dr. Harry K. Fritchman of Boise State College, Boise, Idaho (personal communication) is working on release and settlement of *Allopora petrograpta* larvae in the laboratory, including a histological study of the newly settled planulae. He has found that these larvae are heavily ciliated and capable of considerable muscular contraction. After pushing out of the ampullae, the larvae actively seek the bottom of the dish in the laboratory. If the same behavior obtains in the field, then the larval life of *Allopora* would be very brief. Since the appearance of the first newly settled colonies which were observed in the present study correlated very closely with the first histological observations of empty ampullae in the females, there is probably, at most, a very short planktonic stage. As Thorson (1950) points out, prolonging larval life allows greater mortality from predation and increases the risk of being swept away from suitable areas. Since *Allopora* planulae are lecithotrophic, the motile stages need not feed, but are available for dispersal.

Living in areas where currents and surge are fairly strong, many of the larvae would be swept away from suitable settling sites if the planktonic stage were very long. This suggests that the planulae settle quite soon after release, rather than enter the plankton.

From consideration of the survivorship curves (Fig. 11), it is obvious that any which settled and disappeared between the monthly observation dates could not be counted. Sometimes the initial level of a survivorship curve can be estimated by knowing the fecundity of the females in the population. Unfortunately, *Allopora californica* does not lend itself to determining this. The number of females cannot be determined since the sexes cannot be identified in the field. Further, the distribution of eggs within the female colony is haphazard. Measures of eggs/unit area and eggs/cyclosystem give such widely variable results as to be useless as an estimate of fecundity. From gross examination, however, the numbers of eggs per female colony are on the order of a few hundred.

The curves in Figure 11 show an initial steep drop in the number of surviving colonies, leveling off at 15–18 months. Those that live this long appear to have a good chance of surviving. The principle mortality, then, occurs during the settling stage and in the first year.

Survivorship curves for *Muricea* were calculated by Grigg (1970). If larval life is included, mortality is greater than 99%. If larval life is excluded, a constant mortaility rate is observed. The gorgonians, in contrast to *Allopora californica*, have a long larval planktonic stage, where most of the mortality takes place. In the study on *Muricea*, early settling stages were not so easily observed as they were in *A. californica*, so Grigg's settlement data may be a conservative estimate of the number which actually settled.

Mortality in young colonies of *A. californica* is often the result of competition for space and of the inability of the colonies to withstand sedimentation. The release of larvae coincides with the time of year when the algal cover is at a minimum and is prior to the settling of many attached benthic organisms, allowing a few weeks in which the incipient colonies may obtain a secure foothold. Sedimentation is a year-round problem. Horizontal surfaces are particularly vulnerable to this smothering by sediment. It is apparent that *A. californica* is best suited for surviving on vertical surfaces or in rocky crevices, away from algae, and where the currents and surge keep the rocks free of debris.

In large colonies of *A. californica*, mortality seems to be limited to mechanical abrasion and breakage. No evidence of predation was ever seen. On rare occasions a colony was seen which had died and which was being overgrown by encrusting organisms, but the reason for death was not apparent.

Other workers (Yonge, 1940; Stephenson and Stephenson, 1933; Grigg, 1970) have suggested that damaged Scleractinian and other corals do regenerate. Only Stephenson and Stephenson (1933), who stated that in branching forms regeneration occurs rapidly to fill in the gap and restore symmetry to the colony, elaborate on any mechanism of regrowth. Since the study of regeneration involves repeated visits to the same colony *in situ*, it is not surprising that information on other species is lacking.

It has generally been accepted that new cyclosystems arise by budding in *Allopora* (Mosley, 1876, 1879). But in the present study no evidence has been found to suggest that budding is involved in asexual reproduction. *De novo* origin of new gastrozooids and dactylozooids occurs at the surface of the colony both in normal and regenerating branch tips. The gastrozooid develops quite fully before new dactylozooids begin to form, possibly induced by the presence of the new gastrozooid.

Cyclosystems must constantly redirect their growth to stay at the edge of the colony. As the branch grows, one would expect to find much wider spaces between cyclosystems at the base than at the tips of the branches. Such is the case in *Pocillopora damicornis* (Wainwright, 1963). Although this is true to some extent in *Allopora,* it is evident that new cyclosystems can arise along the sides of a branch. Usually the cyclosystems in any given area are about the same size. From time to time a much smaller cyclosystem is found, suggesting that it is much younger than the rest in the area.

The data obtained in this study provide basic information on the biology of *Allopora californica.* Future work, both in the field and the laboratory, may be aimed at answering such questions as how far the sperm can travel to a female gonophore, how the sperm locate the gonophore aperture, whether the larvae swim or creep to a settling site, and what induces the formation of new cyclosystems.

It is a pleasure to thank Dr. Cadet H. Hand, Jr. and Dr. Ralph I. Smith of the Department of Zoology, University of California, Berkeley, for encouragement and helpful suggestions. Mrs. Emily Reid prepared Figures 1, 2, 10, and 11. Special thanks are due to Mr. Lloyd F. Austin, first, for his advice as Department of Zoology Histological Microtechnician, and second, for his support as the Berkeley Campus Diving Officer. He introduced me to SCUBA diving and showed me its potential as a research tool for a marine biologist. The Diving Safety Program, which he heads, was instrumental in the success of the project. Over 40 U. C. certified divers helped with the project in numerous ways. Miss Penelope Hermes, Mr. Robert Loew, and Dr. John Z. Ostarello deserve special mention for the time they spent working with me as diving partners. I was supported in part during this study by a National Science Foundation Graduate Fellowship.

SUMMARY

1. The life history of the hydrocoral *Allopora californica* has been studied over a two-year period. The formation of male and female gonophores is described and the maturation of sperm and eggs within these is illustrated.

2. Eggs are fertilized within the ampullae in the female colony. Subsequent larval development is described. The yearly cycle of sperm and egg maturation begins in January and culminates with release of planula larvae in November.

3. Field observations to study settlement and mortality of young colonies showed that only a very small percentage of them survive a full year. Competition for space and smothering by sediment are two main factors accounting for the high mortality.

4. The process of regeneration in the hydrocoral was studied using photography in the field combined with histological study in the laboratory. After a branch is

cut the broken end has sharp edges and many damaged cyclosystems. Within a week new tissue has covered the wound, and new cyclosystems are visible after 7–8 weeks. Upward growth resumes 4–5 months after a branch has been cut.

LITERATURE CITED

England, H. M., 1926. Development of gonophores of the Stylasteridae. *Proc. Roy. Soc. London,* **99**: 265–283.

Goedbloed, A. F., 1962. On the structure and development of the gonophores of *Allopora blattea* and *Stylaster roseus. Proc. K. Ned. Akad. Wetensch.* (*Amsterdam*) *Series C.,* **65**: 522–531.

Grigg, R. W., 1970. Ecology and population dynamics of the gorgonians, *Muricea californica* and *Muricea fruticosa* Coelenterata: Anthozoa. *Ph.D. thesis, University of California, San Diego.*

Hickson, S. J., 1888. The sexual cells and the early stages in the development of *Millepora plicata. Phil. Trans. Roy. Soc. London Series B,* **179**: 193–204.

Hickson, S. J., 1890. On the maturation of the ovum and the early stages in the development of *Allopora. Quart. J. Microscop. Sci.,* **30**: 579–598.

Hickson, S. J., 1891. The medusae of *Millepora murrayi* and the gonophores of *Allopora* and *Distichopora. Quart. J. Microscop. Sci.,* **32**: 375–407.

Light, W. J., 1970. *Polydora alloporis,* new species, a commensal spinoid (Annelida, Polychaeta) from a hydrocoral off central California. *Proc. Calif. Acad. Sci.,* **37**(14): 459–472.

Little, G., 1971. Aspects of the biology of the subtidal hydrocoral *Allopora californica* Verrill (1866) with emphasis on reproduction, recruitment and mortaility of new colonies, and regeneration. *Ph.D. thesis, University of California, Berkeley.*

Miller, R. M., 1966. Chemotaxis during fertilization in the hydroid *Campanularia. J. Exp. Zool.,* **161**(1): 23–44.

Mosley, H. N., 1876. Preliminary note on the structure of the Stylasteridae, a group of stony corals which, like the Milleporidae, are hydroids and not anthozoans. *Proc. Roy. Soc. London,* **25**: 93–101.

Mosley, H. N., 1879. On the structure of the Stylasteridae, a family of the hydroid stony corals. *Phil. Trans. Roy. Soc. London Series B,* **169**: 425–503.

Newcomb, W., 1868. Description of a new species of *Pedicularia. Proc. Calif. Acad. Sci.,* **3**: 121–122.

Roth, R. C., 1969. Effects of constant current on *Allopora californica. Senior honors thesis, University of California, Berkeley.*

Stephenson, T. A., and A. Stephenson, 1933. Growth and asexual reproduction in corals. *Gt. Barrier Reef Exped. Sci. Rep.,* **3**(7): 167–217.

Thorson, G., 1950. Reproductive and larval ecology of marine bottom invertebrates, *Biol. Rev.,* **25**: 1–45.

U.S. Navy Diving Manual, 1963. Navships 250–538. Washington, D. C.

Verrill, A. E., 1866. Synopsis of the polyps and corals of the North Pacific Exploring Expedition under Commodore C. Ringgold and Captain John Rodgers, U.S.N., from 1853 to 1856; collected by Wm. Stimpson, naturalist to the expedition; with descriptions of some additional new species from the west coast of North America. Pt. 3: Madreporaria. *Proc. Essex Inst.,* **5**(3): 17–50.

Wainwright, S. A., 1963. Skeletal organization in the coral, *Pocillopora damicornis. Quart. J. Microscop. Sci.,* **104**: 169–183.

Yonge, C. M., 1940. The biology of reef-building corals. *Gt. Barrier Reef Exped., Sci. Rep.,* **1**(13): 353–391.

Zullo, V. A., 1963. A review of the subgenus *Armatobalanus* Hoek (Cirripedia: Thoracica), with the description of a new species from the California coast. *Ann. Mag. Natur. Hist., Series 13,* **6**: 587–594.

Reference: *Biol. Bull.*, **145**: 565–579. (December, 1973)

RECOGNITION OF SYMBIOTIC ALGAE BY *HYDRA VIRIDIS*. A QUANTITATIVE STUDY OF THE UPTAKE OF LIVING ALGAE BY APOSYMBIOTIC *H. VIRIDIS*

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Many invertebrates form stable hereditary associations with unicellular algae. Experimental studies of these associations have dealt generally with the nutritional relationship between algae and host, and specifically with the translocation of carbohydrate from algae to animal (Smith, Muscatine, and Lewis, 1969). There have been very few experimental studies which describe how such associations are established and how the host recognizes potential symbiotic algae. Previous studies have been concerned mainly with specificity, *i.e.*, whether or not a given alga and host can form a stable hereditary symbiosis rather than with actual mechanisms of reinfection. (Whitney, 1907; Goetsch, 1924; Park, Greenblatt, Mattern and Merrill, 1967; Karakashian and Karakashian, 1964; 1965; Bomford, 1965; Hirshon, 1969; Provasoli, Yamasu, and Manton, 1968).

Specimens of *Hydra viridis* have *Chlorella*-like green algae which are hereditary endosymbionts. This symbiotic association offers several advantages for the study of host-symbiont recognition. The hydra can be reared in a defined medium. Asexual budding gives rise to large numbers of animals of similar genetic, nutritional and developmental histories. The algae, which are found in the hydra's endoderm and exclusively in the digestive cells, may be readily separated from the host cells and aposymbiotic hydra may be easily acquired, cultured, and experimentally recombined with algae.

In this paper we describe uptake of symbiotic algae by *Hydra viridis*, the rejection of free-living algae by these hydra, and the events leading to reestablishment of a stable association.

Materials and Methods

Maintenance and culture of organisms

Green specimens of *Hydra viridis* (Florida strain 61) were cultured in "M" solution (Muscatine and Lenhoff, 1965a) under ambient laboratory conditions 22° C, 30 foot candles, 14 hours light and 10 hours dark, and fed daily on freshly hatched *Artemia* nauplii. *H. viridis* occasionally produces algae free eggs. From these eggs we developed a class of aposymbiotic hydra, which we cultured and maintained as above. Algae strains and their sources are listed in Table I. All algal stocks with the exception of *H. viridis* symbionts, were maintained in 1.5% agar slants of Loefer medium (LMM) as modified by Karakashian (1963). Sub-cul-

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TABLE I

Summary of algae used in this study

Strain	Host	Remarks
3H	*Paramecium bursaria*	Isolated and cultured by Karakashian (1963)*
34	*Paramecium bursaria*	Isolated and cultured by Karakashian (1963)*
130C	*Paramecium bursaria*	Isolated and cultured by Loefer (1936)**
42A	*Paramecium bursaria*	Isolated and cultured by Karakashian (1963)*
NC64A	*Paramecium bursaria*	Isolated and cultured by Karakashian (1963)*
838	*Spongilla* sp.	Isolated and cultured by R. A. Lewin**
397	Free-living *Chlorella vulgaris*	**
	Algae from *Hydra viridis*	Isolated en masse from cultures of the host

* Obtained from Dr. Stephen J. Karakashian, State University of New York, College of Old Westbury.

** Obtained from the Indiana University Culture Collection of Algae.

tures of algae were prepared aseptically as needed in 5 ml liquid LMM in 15 ml test tubes at 22° C under the continuous illumination of two 40-watt fluorescent lights situated 15 cm below the bottom of the culture tubes. Five days after inoculation these subcultures yielded approximately 0.15 ml wet packed algae (*ca.* 10^8 cells per ml). Symbionts from *H. viridis* were isolated from about 400 one-day starved hydra as described by Muscatine (1967). This procedure yielded about 6×10^7 algae which were used immediately in experiments.

Preparation of ^{14}*C-labeled algae*

Algae harvested from hydra or obtained from cultures were incubated for 45 min, in 1 ml of "M" solution containing 5 μCi $NaH^{14}CO_3$. Labeled algae were then washed 3 times in "M" solution and an aliquot of cells was withdrawn for assay of radioactivity. Such incubation yielded algae with a relative specific activity of 7×10^5 disintegrations min^{-1} mg^{-1} protein nitrogen. Protein nitrogen was measured by the method of Lowry, Rosebrough, Farr and Randall (1951).

Injection of algae into hydra

The injection apparatus consisted of 0.1 ml capacity Hamilton glass syringe fitted with heavy-walled, 2 mm inside diameter Tygon tubing. The syringe and tubing were filled with mineral oil. The injection pipette was prepared from glass capillary tubing drawn out to approximately 0.15 mm outside diameter at the tip. Cell suspensions (10^7–10^8 cells per ml) were introduced into the pipette and the cells were allowed to settle in the tip. The filled pipette was then inserted into the Tygon tubing, taking care to avoid introduction of air bubbles. At the same time, slight suction was applied with the syringe to prevent algae from being prematurely expelled from the pipette. Before injection, the base of each relaxed, attached hydra was touched with the tip of the pipette causing the hydra to retract into a spherical shape. The tip of the pipette could then be easily inserted into the mouth of the hydra. Once the tip was introduced into the mouth it was held in place for a few seconds to allow the hydra's mouth to close tightly around it. The thick algal suspension was then delivered by slight pressure on the syringe plunger. With practice the injection volume was adjusted so that it inflated the hydra to roughly twice its original size. While it was not always possible to control or even measure the volume of algae injected, more cells were injected than could be taken up by the hydra. Over-inflation was avoided since it caused immediate collapse of the hydra and regurgitation of the algae, regardless of the type of algae injected. Further, when the pipette was removed, the mouth invariably gaped and algae tended to leak out into the medium. To prevent gaping, hydra to be injected were each fed a single *Artemia* nauplius and then injected within a few minutes after the shrimp was swallowed.

Assay of radioactivity in hydra and algae

To measure the quantity of labeled algae taken up by hydra digestive cells, individual injected animals were placed in a drop of "M" solution and slit down one side with a scalpel. Algae not taken up by the digestive cells were flushed from the interior with a fine pipette. The washed hydra and the solution with unincorporated algae were each applied to separate half-inch squares of Whatman 3 mm filter paper, treated with two drops of 0.1 N HCl to remove unused $^{14}CO_2$ and dried under a heat lamp. The dried filter paper squares were assayed for radioactivity in 5 ml scintillation fluid (50 g 2,5-diphenyloxazole, 0.625 g 1,4-bis, 2-5 phenyloxazolyl benzene in 500 ml toluene) using a Nuclear Chicago liquid scintillation counter (Model *Mark I*). Uptake of algae into hydra digestive cells was expressed as a percentage of the total radioactivity in algal cells administered by injection. The possibility existed that soluble ^{14}C-labeled material might move from the algae in the coelenteron to the hydra cells even though the algae themselves might not be taken up. To check this possibility an injection was performed using algae labeled with $Na_2{}^{35}SO_4$. The uptake pattern of these algae did not differ significantly from those labeled with $NaH^{14}C_3$ which was used in all other determinations. In some experiments, whole green hydra and aposymbiotic hydra were incubated in 1 ml "M" solution containing 5 μCi $NaH^{14}CO_3$ for one hour under the continuous illumination of "Gro-Lux" fluorescent lamps delivering about 260 footcandles to the surface of the hydra. Following incubation, the hydra were rinsed, dried on Whatman 3 mm squares, treated with 0.1 N HCl, and counted as described above.

Maceration of hydra

To examine individual hydra digestive cells for the presence of algae and to count their numbers, hydra tissue was macerated on a microscope slide in either

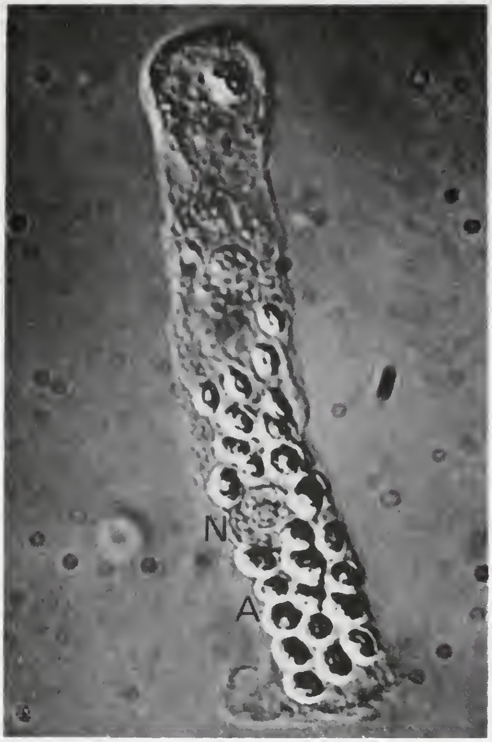

FIGURE 1. Photomicrograph of a typical green hydra digestive cell obtained by maceration and showing the basal lower location of the symbiotic green algae which number 25 in this cell (A, algal cells; N, nucleus of the digestive cell), 936 ×, Nomarski interference microscopy.

0.2% acetic acid and then fixed with a drop of 0.004% osmium tetroxide or a solution consisting of acetic acid, glycerol, and water (1:1:13 v/v) and observed immediately, under a compound microscope at 400 ×. Counts were recorded with a hand tally counter.

RESULTS

To describe the uptake of algae by aposymbiotic hydra, it was necessary first to characterize quantitatively the normal population of algae in green hydra. Three criteria were used: (a) total number of algae per hydra, (b) mean number of algae per digestive cell, and (c) amount of ^{14}C fixed by whole green hydra, shown previously to be proportional to the number of algae present (Muscatine and Lenhoff, 1965b).

Total number of algae in green hydra

Each of ten individual hydra, one-day starved and having one bud, were homogenized with a small tissue grinder in 0.5 ml "M" solution. Aliquots of this

TABLE II

Effect of maintenance conditions on the number of algae per digestive cell in H. viridis. *Animals were maintained at room temperature. Illumination (200 ft-c) was provided by Sylvania Gro-lux lamps. Diurnal room illumination was 10 hrs light (30 ft-c) and 14 hrs dark. Data expressed as average number of algae/digestive cell ± s.d.; N = 100 cells from Zone 2 of five hydra*

Condition	Number of algae Day 2	Number of algae Day 4
Constant light, starvation	21.0 ± 5.7	21.8 ± 4.6
Constant light, fed every 24 hr	20.0 ± 4.7	20.8 ± 4.1
Diurnal light, starvation	20.4 ± 1.0	20.0 ± 1.7
Diurnal light, fed every 24 hr	18.0 ± 2.6	18.3 ± 1.9
Constant dark, starvation	17.0 ± 1.4	11.0 ± 3.0
Constant dark, fed every 24 hr	12.3 ± 3.8	6.8 ± 2.3

homogenate were transferred to a hemacytometer and from the algal count the number of algae per hydra was obtained. Such hydra were found to contain $1.47 \times 10^5 \pm 4 \times 10^4$ algal cells.

Number of algae per digestive cell

Gross examination of whole green hydra revealed that the densest population occurred in the mid-zone of the polyp rather than in the tentacles or stalk. To quantify this distribution, individual hydra were cut transversely into three pieces; hypostome and tentacles (Zone 1); gastric column including the budding region (Zone 2); and the stalk and pedal disc (Zone 3). Pieces from each zone were macerated separately on a microscope slide and the intracellular algae in individual digestive cells were counted. The greatest mean number of algae per digestive cell was observed in cells from Zone 2. In this zone about 18 ± 2.6 algae were encountered in each cell most frequently. Significantly fewer algae per cell (approximately 12) were encountered in Zones 1 and 3. These data show that the algae are distributed throughout the body but the central region contains the highest number of algae per cell.

Figure 1 shows a *Hydra viridis* digestive cell prepared by maceration. The zoochlorellae are located in the proximal portion of the cell. In normal green hydra the algae were always found in this position in digestive cells and never in any other cell types.

It is important to note here that in experiments to be described elsewhere, we found that maintenance of hydra under different light and feeding regimes re-

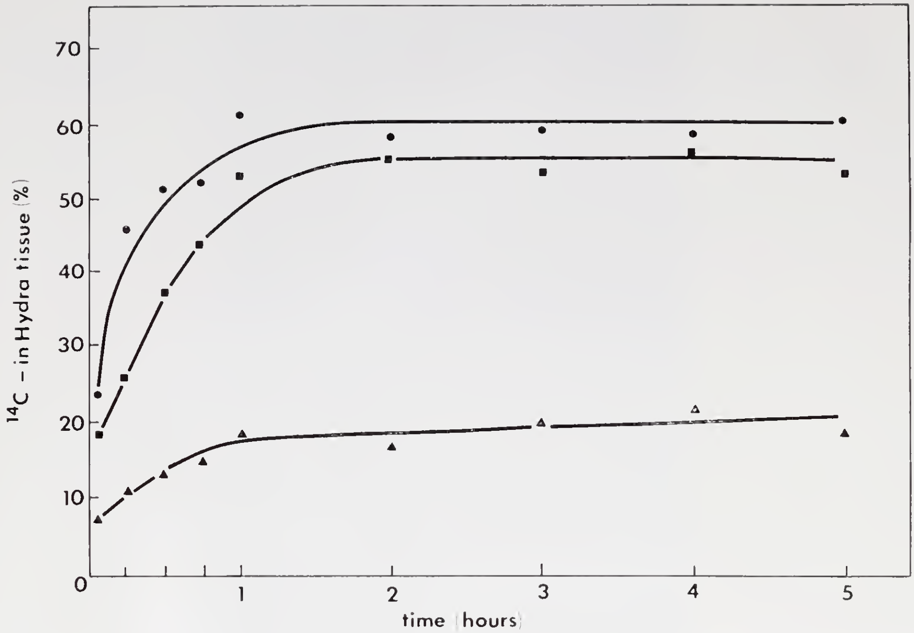

FIGURE 2. Per cent of radioactivity in hydra tissue following time after injection of ^{14}C-labeled algae (open circle, aposymbiotic hydra injected with hydra algae; open square, green hydra injected with hydra algae; open triangle, aposymbiotic hydra injected with NC64A algae; curves fitted by eye).

sulted in different mean numbers of algae per digestive cell, ranging from 7 to 22 as shown in Table II. For example, animals fed daily but kept in darkness had far fewer algae per cell than a hydra fed daily but kept in constant light. Presumably, a hydra fed in darkness grows well enough but its algae do not. We chose to use animals which came from populations fed daily and maintained under ambient diurnal light conditions (30 footcandles, 14 hours light, 10 hours dark). However, data in this paper apply only to animals maintained as described in Methods.

Uptake of algae by aposymbiotic H. viridis

Algae were isolated from normal *H. viridis* and then injected into aposymbiotic *H. viridis.* At various times after injection the hydra were assayed for radioactivity to determine the extent of uptake of algae. Figure 2 shows that the uptake of the radioactively labeled algae is very rapid. Half of the total ^{14}C incorporated by the hydra is taken up in the first two minutes following injection. Uptake of algae by digestive cells continues more slowly thereafter for about one hour and then ceases even though excess algae are still abundant in the coelenteron.

To observe the location and fate of algae after phagocytosis by digestive cells, aposymbiotic hydra were macerated at 30 seconds, 10 minutes, 30 minutes, and finally after 5 hours after injection. Figure 3a shows initial contact of algae and digestive cell. Figure 3b shows that algae are taken up at the distal portion of the cell. About 30 minutes after injection, algae can be found throughout the digestive cell interior, as shown in Figure 3c. Finally after 5 hours, the algae are generally located at the base of the cell, as in normal green hydra digestive cells (3d).

About 5 hours after feeding, *H. viridis* normally regurgitates particulate material not taken up by digestive cells. After regurgitation, the number of algae taken up by aposymbiotic *H. viridis* was determined by maceration and cell counts. Digestive cells of Zone 2 had taken up about $8 \pm 2 (n = 100)$ algae per digestive cell. Since we had previously observed that normal green hydra have 18 ± 2.6 algae per digestive cell (Zone 2), we reasoned that the normal complement of 18 must be obtained by algal cell division. This possibility was investigated by following the course of repopulation.

Repopulation of aposymbiotic H. viridis

Aposymbiotic hydra were injected with unlabeled hydra algae. Five hours later, following regurgitation, and at various times thereafter, the hydra were incubated in $NaH^{14}CO_3$ for one hour and then assayed for fixed ^{14}C. The data in Table III show that the amount of ^{14}C fixed increases most rapidly during the first 168 hours after uptake of algae, and then more slowly until after about 18 days, the amount of ^{14}C fixed is similar to that fixed by green controls. Digestive cells observed at that time had 19 ± 3 algae per cell indicating that repopulation was complete. During the time that repopulation was taking place, the injected hydra fed and produced buds normally. We conclude that following injection and uptake of algae by *H. viridis* the algae undergo relatively rapid cell division until the full complement of algae is obtained.

Uptake of algae by normal green H. viridis

The foregoing data demonstrate that, even if given excess algae, the digestive cells of aposymbiotic *H. viridis* take up only about half of their normal complement of algae, the remainder being acquired by cell division. The data suggest, therefore, that the limitation on uptake of algae is not lack of physical space since ultimately the hydra cells can accommodate 19 algae and in some cases (Fig. 1) as many as 25 per digestive cell. This conclusion was further tested by injecting *H. viridis* algae into normal green hydra. Since the algae injected into hydra were labeled with ^{14}C, they were distinguishable from algae already present in the digestive cells. The curve in Figure 2 shows that green hydra possess the capacity to take up additional algae. Uptake was slower than that of aposymbiotic hydra; half the labeled algae being taken up in about 7 minutes. Digestive cells obtained 5 hours after injection possessed approximately 27 ± 4.9 algae per cell (Zone 2) $(n = 100)$. Since normal green hydra have approximately 18–19 algae per cell, it is evident that roughly 9 extra algae were taken up by digestive cells following injection of algae. This is the approximate number which were taken up by aposymbiotic hydra. The subsequent fate of these extra algae has not yet been determined.

Injection of foreign algae into aposymbiotic H. viridis *and other* Hydra *spp.*

We attempted to infect *H. viridis* aposymbionts and other *Hydra* species with various species of foreign algae, both symbiotic and free-living. Six strains of

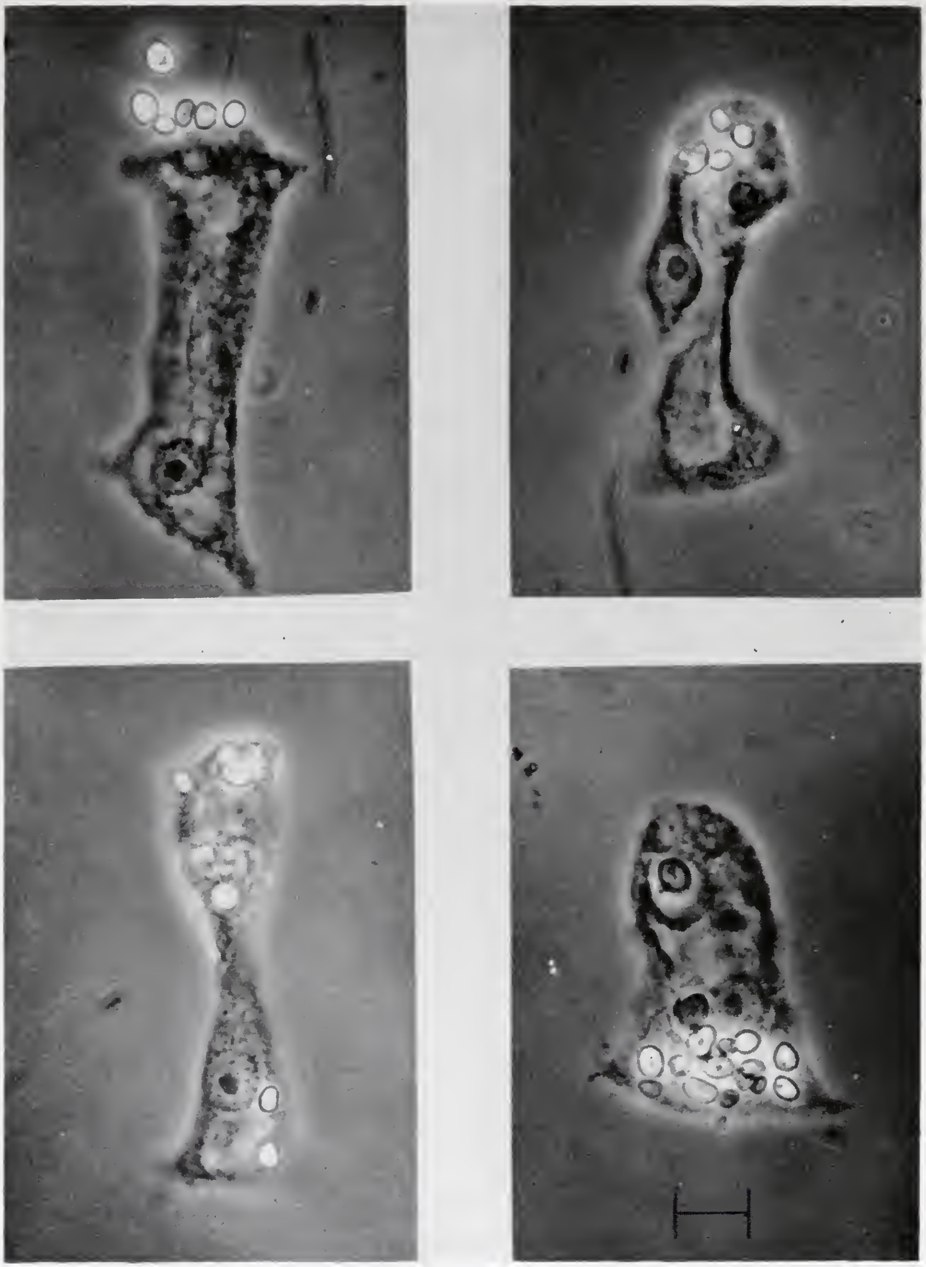

FIGURE 3. Phase contrast photomicrographs showing digestive cells macerated from aposymbiotic hydra at various times after injection of hydra algae. Phase optics employed to distinguish algae (+ birefringence) from other cell organelles and inclusions. Cells oriented with distal absorptive ends up (N, nucleus of animal cell; A, algal cells); starting at upper left, (a) 30 sec after injection, showing algae in contact with distal portion of digestive cell (note

TABLE III

Radioactivity (disintegrations min^{-1}*) in whole hydra due to* $^{14}CO_2$ *fixation by intracellular algae at various times after injection with normal hydra algae. Counts are the average of three hydra. Green control shows the level of* $^{14}CO_2$ *incorporated by algae in standard green hydra. Non-injected aposymbiotic control indicates the level of heterotrophic fixation of* $^{14}CO_2$ *by hydra*

Time after injections	Disintegrations min^{-1}
0.2 (days)	91
1	110
2	145
3	147
6	164
7	179
18	210
32	203
H. viridis (control)	218
Aposymbiotic hydra	15

symbiotic algae, five from *P. bursaria* and one from *Spongilla* sp. and one species of free-living *Chlorella* were tested. Injection of *H. viridis* algae into aposymbiotic hydra served as a control. Success of infection was determined from observations on macerated cells and ability to fix $^{14}CO_2$. The results in Table IV indicate that of the algae injected, only algae from *H. viridis* and one strain of algae from *P. bursaria* were taken up by aposymbiotic *H. viridis*. All other strains of algae were regurgitated almost immediately (5–10 minutes) by some individuals, and in all cases within 5 hours after injection. Those algal strains taken up by *H. viridis* aposymbionts were also injected in nonsymbiotic hydra. There was no evidence of uptake of these algae by cells from these animals (*H. littoralis* or *H. pseudoligactis*). Figure 2 shows that the rate of uptake of NC64A algae by *H. viridis* is slower than that of *H. viridis* algae, with half of the total ^{14}C incorporated being taken up in 10 minutes.

We have found that those aposymbiotic hydra injected with NC64A algae have slightly fewer algae per cell (approximately 15) 60 days after injection than aposymbiotic hydra controls 30 days after injection (approximately 20). Figure 4 shows that the growth rate of hydra infected with NC64A is virtually identical with that of normal green hydra under identical conditions of feeding. We concluded from these data that NC64A algae can establish a successful symbiosis with *H. viridis* although the rate of uptake of the algae, the rate of algal cell repopulation, and the final population density is less than those of normal hydra algae.

DISCUSSION

The results of these experiments show that aposymbiotic *Hydra viridis* can be reinfected after receiving an injection of its normal symbiotic algae or one strain of

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flagellum); (b) 10 min, algae are now within the digestive cell as a result of phagocytosis; (c) 30 min, location of algae shifting from distal to proximal (basal) region; (d) 5 hr, Algae now in normal basal location (*cf.* Fig. 2). Scale bar represents 10 microns.

TABLE IV

Comparison of various algae in their ability to be taken up by specimens of aposymbiotic H. viridis; (–) *not taken up,* (+) *taken up*

Algal strain	Host	H. viridis
3H	*Paramecium bursaria*	–
34	*Paramecium bursaria*	–
130c	*Paramecium bursaria*	–
42	*Paramecium bursaria*	–
NC64A	*Paramecium bursaria*	+
838	*Spongilla* sp.	–
hydra	*Hydra viridis*	+
397	Free-living *C. vulgaris*	–

(symbiotic) algae from *Paramecium bursaria.* On the other hand, aposymbiotic *H. viridis* rejects other strains of *P. bursaria* algae, sponge algae, and free-living algae. These observations, together with the fact that *H. pseudoligactis* and *H. littoralis* reject symbiotic algae (NC64A, *H. viridis* algae) suggest that the establishment of symbiosis in aposymbiotic *H. viridis* is a function of properties of both the algae and the hydra and that the discrimination between them leading to either uptake or rejection may be referred to legitimately as a cellular recognition phenomenon.

Four general phases may be distinguished in the successful establishment of the *H. viridis* symbiosis: (1) A brief period of *contact* and *recognition* followed by (2) *phagocytosis* which may last up to one hour, (3) intracellular transport lasting at least five hours, and finally, (4) a period of algal *growth,* initially relatively rapid, lasting up to two weeks.

Aposymbiotic *H. viridis* becomes reinfected only when the algae are introduced into the colenteron (Goetsch, 1924; Whitney, 1907; Park *et al.,* 1967), presumably because contact between algae and digestive cells is required to initiate the recognition mechanism. We tentatively rule out such physiological parameters as oxygen production by the algae as a significant feature of the recognition mechanism leading to acceptance since all algae produce oxygen but most are still rejected. Similarly, the release of soluble extracellular carbohydrate by symbiotic algae (Smith, *et al.,* 1969) may be ruled out as a chemical cue leading to recognition since all but one of the symbionts were rejected. It would be difficult to explain any specificity in the recognition mechanism based on oxygen production or release of carbohydrate. Current models of cell recognition are based generally on the interaction of molecules at cell surfaces (Smith and Good, 1969). In this connection we note that Slautterback (1967) has detected and described "coated vesicles" in the apical cytoplasm of digestive cells of *Hydra oligactis.* These vesicles are involved in selective phagocytosis of particulate material. They are coated with a highly ordered array of electron dense "pegs" and "globules." As such they emerge as a morphological entity worthy of consideration as possible "recognition sites," that is contingent, of course, on whether or not they occur in *H. viridis.*

Data in Figure 2 show that when the algae are recognized as acceptable symbionts, their uptake into digestive cells is relatively rapid, the process being nearly

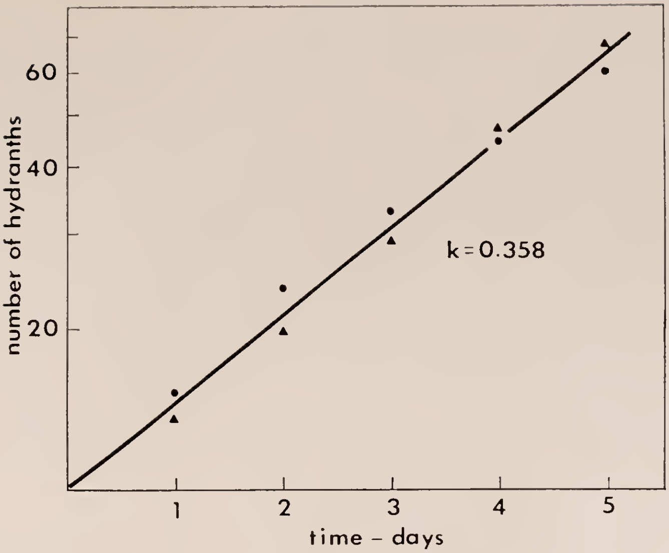

FIGURE 4. Growth rate of green hydra (open triangle with normal algal flora and aposymbiotic hydra artificially infected with NC64A algae (open circle).

completed in less than an hour. These uptake rates compare favorably with those reported for the uptake of *Chlorella* by *P. bursaria* (Hirshon, 1969). It is evident that uptake of algae is accomplished by phagocytosis and results in the formation of intracellular vacuoles but the exact mechanism has not yet been determined. Symbiont vacuoles are described in studies on the fine structure of green hydra by Wood (1959), Oschman (1967), and Park *et al.* (1967). In *Paramecium bursaria* the route of infection is uncertain but algae are believed to be ingested into large vacuoles resembling food vacuoles. At this point foreign algae are sorted out and expelled and symbiotic algae are retained in inidividual vacuoles (Karakashian and Karakashian, 1965; Karakashian, Karakashian and Rudzinska, 1968). When symbiotic algae undergo cell division, their vacuole may temporarily contain several autospores, but these are ultimately disposed into new individual vacuoles (Oschman, 1967; Karakashian *et al.*, 1968).

Of the several factors which may limit the uptake of algae by *H. viridis* to 8 ± 2 cells, the supply of algae and the space available for them can be ruled out on the basis of our observations. Rather, the limiting factor is probably the phagocytosis process itself. The metabolic basis of phagocytosis is fairly well understood. It is temperature and pH dependent and requires energy. Lipid and protein synthesis are required for the formation of vacuolar membranes. (Rowley, 1962; Karnovsky, 1962; Jacques, 1969). We speculate therefore that uptake of algae is limited by the ability of the cells to synthesize vacuolar membranes. The extent to which the presence of food in the coelenteron influences the recognition and uptake of algae has not yet been investigated in detail. Our unpublished observations suggest that the presence of food is not required for uptake of algae but food may compete for phagocytic vacuole membranes which might otherwise be used to take up algae. Neither the stimulus for intracellular transport of algae, its mechanism, nor its selective advantage is yet known.

Little is known of the population dynamics of symbiotic algae. It is generally conceded that the growth rates of algae and host are somehow linked to keep the symbiont population at an optimum level. Experiments in our laboratory suggest that algal growth rates and standing crops may be modified temporarily by environmental factors such as light and nutrients (see also Oschman, 1967; Weis, 1969). For example, green hydra fed in darkness outgrow their algae to the extent that there are only a few algae per digestive cell (Table II). When these hydra are returned to the light the algae immediately undergo rapid growth and within a few days the population is established at an optimum level. Similarly, after injection of algae, aposymbiotic hydra have relatively few algae per cell. Table III shows that within a few days the algae undergo relatively rapid growth until the full complement of algae is attained. These data show that the ability of the symbionts to grow at high maximum intrinsic rates is important in maintaining the population at an optimum level. The factors which contribute to the onset and cessation of these rapid algal growth rates are still unknown.

Associated with the problem of growth is the problem of distribution of algae within a given host. We assume that because we supply excess algae to aposymbiotic hydra, that all digestive cells obtain at least a few symbionts. This is not necessarily true. In fact, small numbers of algae injected into an aposymbiotic hydra would satisfy only a few digestive cells. How do the rest of the digestive cells acquire symbionts from this initial small inoculation? There are at least three possibilities. (1) The algae move from cell to cell. This appears unlikely especially in view of the observation of Burnett and Garofalo (1960) of the fate of bits of green hydra tissue grafted onto albino hydra. (2) The algae are expelled into the coelenteron and then immediately recaptured by other digestive cells. This possibility has not yet been experimentally tested. (3) The remaining aposymbiotic cells do not become infected, rather the rate of cell division of green (algae-laden) digestive cells is faster than aposymbiotic cells and the green cell line becomes dominant as all cells undergo their normal turnover in the hydra. This alternative is consistent with the fact that green hydra grow faster than aposymbiotic hydra particularly when food is limiting (Muscatine and Lenhoff, 1965b).

The rejection of foreign algae by *H. viridis* is also intriguing. Unlike *P. bursaria* where the rejection of foreign algae is perhaps decided after the algae are within a vacuole, rejection by algae by *H. viridis* is apparently decided at the cell surface and leads to a coordinated response involving the whole hydra. Not only is mucus secreted locally to bind the foreign algae together but increased flagellar and muscular activity is elicited and in some cases the algae are gathered up and pushed toward the hypostome by movements of the body in preparation for expulsion only minutes after they are injected into the coelenteron. The ecological importance of rejection should not be underestimated. It is highly likely that in nature *Hydra* feeds on herbivorous crustaceans which themselves have recently ingested unicellular algae. The result is that foreign algae are introduced into their enterons when the crustaceans are digested. The fate of such algae is unknown but the fact that foreign algae are rejected argues against the likelihood that hydra routinely digests algae. Karkashian *et al.* (1968) argue that part of the successful maintenance of algae inside cells prone to autophagy may be their ability to resist lysosomal digestive activity. The algal vacuoles in *Paramecium bursaria* were not observed in association with lysosomes. In contrast, Oschman (1966) has observed "Phagolysosomes" fusing with algae vacuoles. He interprets this to mean that *Hydra* probably digests its algae either wholly or in part under appropriate circumstances. Despite inferences from electron microscopy, conclusive evidence for digestion of algae (*i.e.,* enzymatic hydrolysis of algal consituents) is still lacking.

Infection of aposymbiotic hydra with foreign algae has been reported by Goetsch (1924) and Park *et al.* (1967). In each case the free-living alga *Oocystis* was taken up by the aposymbiotic *H. viridis*. However, in both instances the infection was transitory and the algal population did not reach the level normally found in green hydra. We did not attempt to reinfect *H. viridis* with *Oocystis* and tested only one species of free-living algae which was rejected, and so we regard any conclusions about specificity of infection of *H. viridis* as tentative. However, the infection of *H. viridis* by strain NC64A from *P. bursaria* showed no tendency to decline even though the population densities were slightly less than those in normal green hydra. It thus appears that a persistent symbiosis between *Hydra viridis* (Florida strain 61) and NC64A can be attained. Since NC64A is not a natural symbiont of Hydra, it is not unexpected that its kinetics of uptake and growth rate are different from those of normal symbionts.

The re-establishment of a symbiosis with algae is not unique to *Hydra*. Trench (1969) has shown that aposymbiotic *Anthopleura elegantissima*, a sea anemone, may be re-infected by repeated injection of their normal dinoflagellate symbionts. Provasoli *et al.* (1968) has brought about the re-synthesis of algal symbiosis in *Convoluta roscoffensis*, an acoel flatworm. *Paramecium bursaria* can acquire and maintain other algal strains as well as its native symbionts (Bomford, 1965; Karakashian and Karakashian, 1965; Hirshon, 1969) but in most cases the native symbionts exhibit greater persistence and survival in the host.

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SUMMARY

1. An individual *Hydra viridis* (Florida Strain) harbors approximately 1.5 × 10^5 unicellular green algae. These algae are normally found in the basal portion of gastrodermal digestive cells. An average of approximately 18 algae per digestive cell is encountered in the central region (stomach and budding zone) of the hydra under a given set of maintenance conditions.

2. The average number of algae per digestive cell may range from 7 to 22 depending on feeding schedules and photoperiod under which hydra cultures are maintained.

3. The symbiosis can be synthesized artifically by injecting symbiotic algae from *H. viridis* into the coelenteron of aposymbiotic *H. viridis.* Within 15 minutes after injection the algae are taken up by the digestive cells, apparently by phagocytosis. During the next hour, the algae move from the site of uptake to the basal part of the digestive cell.

4. Both green and aposymbiotic *H. viridis* can take up only about 8 ± 2 algae from an injected suspension of cells. Uptake may be limited by the extent to which the cell can sustain phagocytosis, rather than by availability of intracellular space. In aposymbiotic *H. viridis*, the full complement of 18 algae is attained by algal cell division and requires about 18 days.

5. An assortment of free-living and symbiotic algae were injected into *H. viridis* aposymbionts. All were rejected immediately with the exception of hydra algae and a symbiotic alga from *Paramecium* (NC64A) which formed a stable hereditary endosymbiosis with *H. viridis.* Growth rates of these hydra were virtually identical with those of normal green hydra.

6. The acquisition of algae by hydra digestive cells appears to involve several "phases" including contact, recognition, phagocytosis, and intracellular transport.

LITERATURE CITED

BOMFORD, R., 1965. Infection of algae-free *Paramecium bursaria* with strains of *Chlorella, Scenedesmus,* and a yeast. *J. Protozool.,* **12**: 221-224.

BURNETT, A., AND M. GAROFALO, 1960. Growth pattern in the green hydra, *Chlorohydra viridissima. Science,* **131**: 160-161.

GOETSCH, W., 1924. Die symbiose der Susswasser-hydroiden und ihre kunstliche Beeinflussung. *Z. Morphol. Okol. Tiere,* **1**: 660-731.

HIRSHON, JORDON B., 1969. The response of *Paramecium bursaria* to potential endocellular symbionts. *Biol. Bull.,* **136**: 33-42.

JAQUES, P. J., 1969. Endocytosis. Pages 395-420 in J. T. Dingle and Honor B. Fell, Eds., *Lysosomes in Biology and Pathology,* Volume 2. North-Holland Publishing Co., London.

KARAKASHIAN, J., 1963. Growth of *Paramecium bursaria* as influenced by the presence of algal symbionts. *Physiol. Zool.,* **36**: 52-67.

KARAKASHIAN, M. W., AND S. J. KARAKASHIAN, 1964. The inheritance of susceptibility to free-living algal infection in aposymbiotic *Paramecium bursaria. J. Protozool.,* **11** (suppl.): 19.

KARAKASHIAN, S. J., AND M. W. KARAKASHIAN, 1965. Evolution and symbiosis in the genus *Chlorella* and related algae. *Evolution,* **19**: 368-377.

KARAKASHIAN, S. J., M. W. KARAKASHIAN AND M. RUDZINSKA, 1968. Electron microscopic observations on the symbiosis of *Paramecium bursaria* and its intracellular algae. *J. Protozool.,* **15**: 113-128.

KARNOVSKY, M. L., 1962. Metabolic basis of phagocytic activity. *Physiol. Rev.,* **42**: 143-168.

LOEFER, J. B., 1936. Isolation and growth characteristics of the zoochlorella of *Paramecium bursaria. Amer. Natur.,* **70**: 184-188.

LOWRY, O. H., N. ROSEBROUGH, A. FARR AND R. RANDALL, 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.,* **193**: 265-275.

MUSCATINE, L., 1967. Symbiosis of hydra and algae. III. Extracellular products of the algae. *Comp. Biochem. Physiol.,* **16**: 77-92.

MUSCATINE, L., AND H. M. LENHOFF, 1965a. Symbiosis of hydra and algae. I. Effects of some environmental cations on growth of symbiotic and aposymbiotic hydra. *Biol. Bull.,* **128**: 415-424.

MUSCATINE, L., AND H. M. LENHOFF, 1965b. Symbiosis of hydra and algae. II. Effects of limited food and starvation on growth of symbiotic and aposymbiotic hydra. *Biol. Bull.,* **129**: 316-328.

Oschman, J. L., 1966. Apparent digestion of algae and nematocysts in the gastrodermal phagocytes of *Chlorohydra viridissima*. *Amer. Zool.*, **6**: 320.

Oschman, J. L., 1967. Structure and reproduction of the algal symbionts of *Hydra viridis*. *J. Phycol.*, **3**: 221–228.

Park, H., C. L. Greenblatt, C. F. T. Mattern and C. K. Merrill, 1967. Some relationships between *Chlorohydra*, its symbionts and some other chlorophyllous forms. *J. Exp. Zool.*, **164**: 141–162.

Provasoli, L., T. Yamasu and I. Manton, 1968. Experiments of the resynthesis of symbiosis in *Convoluta roscoffensis* with different flagellate cultures. *J. Mar. Biol. Ass. U.K.*, **48**: 465–479.

Rowley, D., 1962. Phagocytosis. *Advan. Immunol.*, **2**: 241–264.

Slautterback, D. B., 1967. Coated vesicles in absorptive cells of *Hydra*. *J. Cell Sci.*, **2**: 563–572.

Smith, D. C., L. Muscatine and D. H. Lewis, 1969. Carbohydrate movement from autotrophs to heterotrophs in parasitic and mutualistic symbiosis. *Biol. Rev.*, **44**: 17–90.

Smith, R. T., and R. A. Good, 1969. *Cellular Recognition.* Appleton-Century-Crofts, New York, 328 p.

Trench, R., 1969. The physiology and biochemistry of zooxanthellae symbiotic with marine coelenterates. III. The effects of homogenates of host tissues on the excretion of photosynthetic products *in vitro* by zooxanthellae from two marine coelenterates. *Proc. Roy. Soc. London Series B*, **177**: 251–264.

Weis, D., 1969. Regulation of host and symbiont population size in *Paramecium bursaria*. *Experientia*, **25**: 664–666.

Whitney, D. D., 1907. Artificial removal of the green bodies of *Hydra viridis*. *Biol. Bull.*, **13**: 291–299.

Wood, R. L., 1959. Intercellular attachment in the epithelium of hydra as revealed by electron microscopy. *J. Biophys. Biochem. Cytol.*, **6**: 343–352.

Reference: *Biol. Bull.*, **145**: 580–588. (December, 1973)

THE ECOLOGY OF *CHONDRUS CRISPUS* AT PLYMOUTH, MASSACHUSETTS. III. EFFECT OF ELEVATED TEMPERATURE ON GROWTH AND SURVIVAL[1]

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The specific relationship between temperature and the responses of attached marine algae has had limited attention. Of particular current interest is the effect of elevated temperatures on growth, reproduction, and survival of populations of algae at locations where additions of heat are proposed in the coastal environment. Ignatiades and Smayda (1970), Ott (1966) and Provasoli (1963) found that temperatures of 25°–30° C were frequently above optimum for growth of cold water marine algae in culture. Adey (1970) showed that growth of boreal subarctic *Lithothamniae* declined with temperatures above 10°–15° C. Studies by Ehrke (1931) of respiration and photosynthetic rates with *Fucus, Enteromorpha,* and *Delesseria,* and by Kanwisher (1966), Mathieson and Burns (1971), and Newell and Pye (1968) with *Chondrus* demonstrate that thermal injury to the metabolism of these species occurs at temperatures above 26° C. Ring (1970) noted that holdfasts of *Chondrus* died in culture within two days at 29° C, but the erect shoots survived for two months at this temperature. Growth of the erect shoots was better, however at 10° than at 15° C. Burns and Mathieson (1972) found that sporeling growth increased with an increase in temperature up to 19° C. In comparison, Newton, Devonald, and Jones (1957) failed to find a respiratory maximum for *Chondrus* even at 35° C.

These data do not allow for confident predictions, even approximate, on the effect of thermal additions on populations of attached marine algae. A nuclear thermoelectric generating plant is under development at Rocky Point, Plymouth, Massachusetts (Prince and Kingsbury, 1973b). Its thermal discharge is located in the midst of one of the principal commercial beds of *Chondrus crispus* on the western Atlantic coast. The purpose of this study was to culture *Chondrus* under controlled conditions at a growth rate approximating that in nature and to determine the effects of elevated and depressed temperatures on growth and survival. We have reported the growth and reproductive responses of the natural population at Plymouth in the other papers of this study (Prince and Kingsbury, 1973a, 1973b).

MATERIALS AND METHODS

Three series of culture experiments were conducted between January and December, 1970, to establish normal growth curves, to identify optimum conditions,

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and to perfect techniques of culture, experimentation, and examination. These experiments also yielded information on growth of sporelings at temperatures and light conditions above and below optimum. In this work it became necessary to move culture facilities from Ithaca, New York, to New London, Connecticut. Seawater for culture media was obtained from several locations and handled in different ways. Lighting conditions and measurements of light intensity also varied as techniques were refined. For these reasons, results of the three series are not exactly comparable. We describe here methods and results for the third series of experiments, the most refined, with comments on the preceding two series only where results amplify those of the third series. Following this work, we did additional sets of experiments to look more closely at specific lethal or deleterious characteristics of elevated temperatures.

Culture chambers were illuminated with dual 40 watt cool white fluorescent tubes (Westinghouse F40CW). Intensity was regulated by varying the distance of the cultures from the tubes, covering one of the tubes, or interposing a plastic screen filter. Intensity was determined in m watts/cm^2 (Yellow Springs Radiometer, No. 65, probe 6551 set on a large heat sink, the whole covered by a petri dish to give readings equivalent to those inside a culture flask). Several readings were taken on each shelf and averaged.

Temperature in the culture cubicles was monitored by bimetallic, Fahrenheit recorders (Bachrach Tempscribe) checked by a centrigrade mercury thermometer. Temperatures were held to within ±0.6° C of desired, with a diurnal fluctuation of 1.0° C as the lights went on and off. Most experiments were conducted under a light regimen of 16 hours light, 8 hours dark (16:*8*); some at 10:*14*.

Culture medium was constructed with natural seawater, filtered (Whatman No. 1), and enriched with nitrate, phosphate, soil extract, and a vitamin and trace metal solution based upon Guillard's medium "f" (Guillard and Ryther, 1962) at half strength. The composition of the culture medium was as follows: KNO_3 72.2 mg, KH_2PO_4 8.0 mg, (the latter two based upon Iwasaki, 1961), $CuSO_4 \cdot 5H_2O$ 0.01 mg, $ZnSO_4 \cdot 7H_2O$ 0.022 mg, $CoCl_2 \cdot 6H_2O$ 0.01 mg, $MnCl_2 \cdot 4H_2O$ 0.18 mg, $NaMoO_4 \cdot 2H_2O$ 0.006 mg, Fe (as Ferric sequestrine, 13% iron; Geigy Indust. Chemicals, New York) 5 mg, Thiamine 0.2 mg, B_{12} 1 μg, Folic Acid 1 μg, Biotin 1 μg, Riboflavin 0.25 μg (based upon Provasoli, 1964), soil extract 50 ml, seawater (aged at least 2 months) to one liter. The seawater used in the third series of experiments was collected at the University of Connecticut's Marine Station at Noank in March, 1970, and was aged seven months before use. Its salinity varied between 29 and 31‰; that of the medium made from it was 1‰ less. The pH of the medium varied between 8.1–8.3. All media were sterilized before use by autoclaving. Precipitation problems were minimized by using aged seawater, and very slow cooling.

Reproductive plants were collected at Rocky Point and Manomet Point (Prince and Kingsbury, 1973b) in seawater, iced during transport to Connecticut, and refrigerated (0.5°–3.0° C) overnight. The following morning, tips with apparently ripe carposporic or tetrasporic sori were excised from the cleanest and least epiphytized plants, washed with jets of sterile seawater at 12° C or scraped with a coarse artist's brush and forceps, and placed in petri dishes of sterile seawater at 11.7° C. Spore release sometimes occurred on the same day as preparation of the tips, possibly in response to temperature variations in handling, but was more usual on the second to fourth days. Tips not discharging by the fourth day were discarded. The discharged spores form a pink-red spot under the tip and must be gathered within 2–4 hours after release or they become attached too firmly to each other and the substrate for removal. Fresh spore masses were picked up with a Pasteur pipette, sprayed over a glass side in a petri dish of sterile seawater, and allowed to settle for 0.5 hours after which the spores were firmly attached to the slide. These manipulations were carried out in a room at 21° C. The temperature of the seawater medium rose during the period of manipulation from 12° to approximately 18° C before the cultures were placed in the cubicles.

Growth of sporelings was recorded as a function of the diameter of the holdfast. Three slides were examined per spore type (tetraspore or carpospore), experimental condition, and age, and 15–40 holdfasts were measured per slide. Growth curves were fitted by inspection to a semi-log plot of sporeling area *vs*. time. Doubling of area per day (K) was calculated:

$$K = \ln \frac{A_2}{A_1}\left[\frac{1}{(T_2 - T_1)\ln 2}\right]$$

where A_2 and A_1, the areas of the sporelings at times T_2 and T_1 respectively, were selected within the interval of the logarithmic phase of growth.

For examination, aqueous mounts were made of the experimental slides directly, using seawater at the same temperature as the experimental conditions (except that water at 11.7° C was used for cultures maintained at 4.4° C to avoid coverglass fogging). Measurements, at 100 or 430 magnification as necessary, were accomplished within 15–30 minutes per slide in a 20° C room. Slides were restored to culture conditions immediately after examination.

By experimental series III techniques had evolved to the point where contamination of cultures was a minor problem. Diatoms were the most frequent contaminants. Slides containing large numbers of sporelings (*ca*. 200) were occasionally washed with a jet of sterile water to remove any percipitated medium or diatoms. Others were handled only if necessary. In those few cultures which developed persistent contamination with diatoms, it could be kept under control by weekly washing with sterile water and an artist's brush. This treatment may have removed dead or poorly attached spores of *Chondrus*, but had no apparent effect on established germlings. In the initial series of experiments, germanium dioxide (10 mg/l) was used to eliminate diatom contamination (Lewin, 1966), without apparent effect on the morphology or growth rate of young *Chondrus* plants.

The exact time at which an organized, multicellular holdfast or erect shoot dies is difficult or impossible to determine. Mortality of spores is easier to recognize as an event in time. Experiments on lethal or injurious effects of elevated temperatures therefore were conducted with freshly released, apparently healthy spores. We have described the morphology and cytology of normal spores and spore germination elsewhere (Prince and Kingsbury, 1973a). Death of spores is characterized most conspicuously by loss of color and rhodoplast organization.

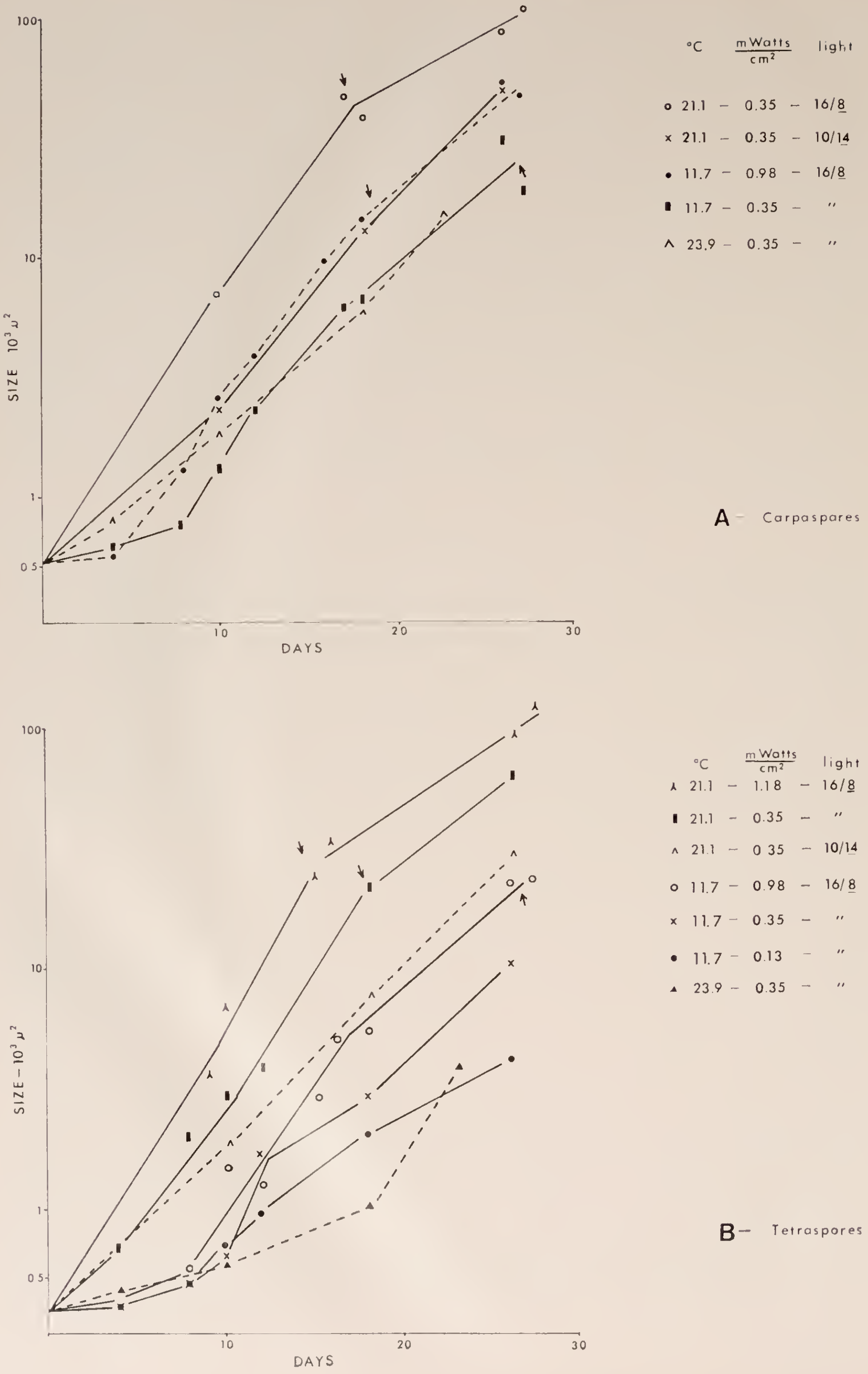
In the first set of mortality experiments, cultures were initiated in the same manner as described above. After a half hour in the 20° C preparation room to allow settling and attachment, cultures were transferred to culture rooms and grown at 0.35 m water/cm^2, 16:*8* photoperiod, at 11.7°, 21.1°, and 23.9° C. After four days, 10–15 separate fields were counted for each culture, and at least 3 cultures were counted per spore type and temperature. Proportion of dead to living spores was recorded and analyzed statistically. In the second set of these experiments, intended to simulate the effects of sudden exposure to a thermal plume, handling was the same except that the spores were given a brief period of thermal elevation while held in pipettes before being sprayed on the culture slides. Thermal "shock" periods of 0.5, 1.0, or 6.0 minutes were administered by immersion of the sealed pipette tip in a water bath. After the shock period, spores were sprayed over a slide in media at 21° C, held for 0.5 hours at this temperature, then cultured at 11.7° C, 0.35 m watts/cm^2, and 16:*8* light. The interposition of 0.5 hours at 21° C was intended to reproduce the effect of thermal effluents in which subsequent cooling would not be instantaneous. Controls were subjected to the same intermediate warm period. Condition of spores was determined four days after the "shock" treatment, recorded and analyzed as above. A third set of experiments was conducted with fully developed plants held at 26.7° C for an extended period, other conditions similar to the above.

RESULTS

Chondrus spores germinate and grow well in culture at temperatures similar to those found in the field. An initial lag phase (Fig. 1A and B) is followed by a logarithmic phase of growth. Initiation of the erect shoot from the original sporeling holdfast (Prince and Kingsbury, 1973a) is accompanied by diminution in the growth rate.

During the lag phase, single-celled and few-celled sporelings predominated. Little increase in diameter of the sporeling accompanied the first divisions; subsequent increase was marked and regular. The presence and extent of the lag phase appears to be related to spore type, light intensity, and particularly temperature. We do not have enough points to establish the duration of a lag phase under the most rapid development characteristic of optimum conditions.

The termination of the logarithmic phase of growth of the sporeling holdfast coincided with organization and appearance of the erect shoot from it (arrows, Fig. 1A and B). In this reorganization of growth pattern, major meristematic activity is transferred from the rim of the holdfast to the tip of the newly forming shoot. In experiments of the earlier series carried to day 51, 92–100% of the carpospore-derived sporelings produced erect shoots, but only 72–87% of the tetraspore-derived holdfasts had done so under the same cultural conditions. Initiation of erect shoots by sporelings under equivalent conditions appeared to be primarily a function of size of the holdfast. Sporelings grown under optimal conditions produced erect shoots significantly earlier than did sporelings grown under suboptimal conditions. For example, in one experiment, tetraspore-derived holdfasts grown at 11.7° C required an average 26 days before erect shoot formation; those at 21.1° C formed erect shoots on the 18th day. The threshold size of holdfasts in these experiments appeared to be about 10×10^3 μ^2. Under suboptimal photoperiod (10:*14* instead of 16:*8*), however, tetraspore- and carpospore-derived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x 21.1 – 0.35 – 10/14
• 11.7 – 0.98 – 16/8
▮ 11.7 – 0.35 – ″
^ 23.9 – 0.35 – ″
A – Carpospores
100
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SIZE – $10^3 \mu^2$
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DAYS
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⅄ 21.1 – 1.18 – 16/8
▮ 21.1 – 0.35 – ″
^ 21.1 – 0.35 – 10/14
o 11.7 – 0.98 – 16/8
x 11.7 – 0.35 – ″
• 11.7 – 0.13 – ″
▲ 23.9 – 0.35 – ″
B – Tetraspores

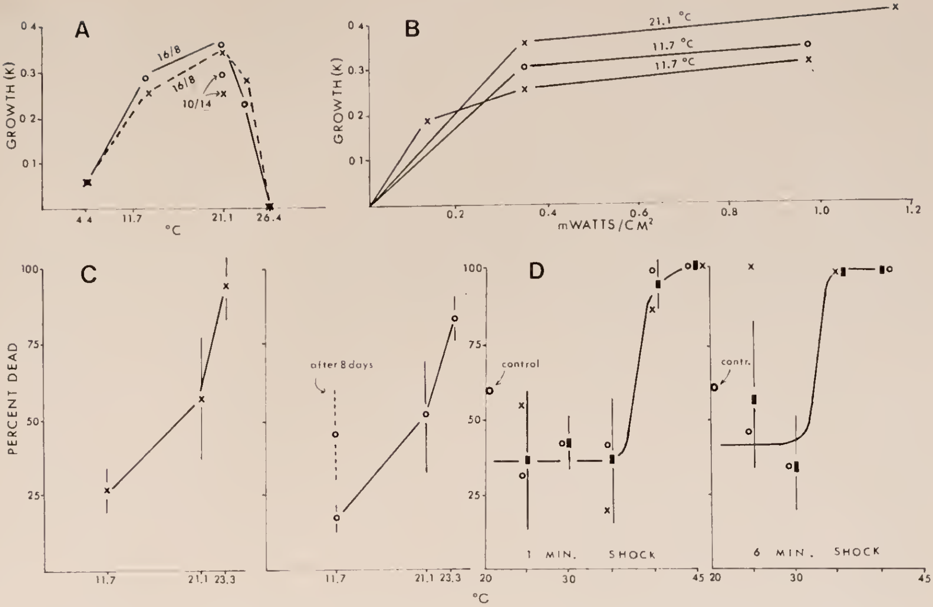

FIGURE 2. (A) Effect of temperature on growth rate of germlings. Curves are for light regimen 16:*8;* two additional points for 10:*14* also shown; in all figures: x, tetraspores or tetraspore-derived germlings; o, carpospores or carpospore-derived germlings; (B) effect of light intensity on growth rate of germlings at two temperatures; (C) mortality of spores after 4 days at elevated temperatures; (D) mortality of spores after brief shock treatment with elevated temperatures; curves by inspection.

sporelings which exceeded this size had not formed erect shoots by the end of the experiment.

Under our experimental conditions, temperature presented the greatest effect on growth during the logarithmic phase (Fig. 1A and B). Some experiments in the earlier series (not graphed) were conducted at 4.4° C. At that temperature, germination and growth of tetraspore- and carpospore-derived sporelings were severely inhibited. After 19 days the cultures still consisted mainly of undivided spores; only a few 2–4 celled sporelings were present. After 25 days a few small holdfasts had developed, but undivided spores were still in the majority. At the other extreme (also not graphed), cultures maintained at 26.7° C displayed signs of injury and death, and did not grow. All carpospores were dead within 4 days; all tetraspores within 4–10 days in experimental series III. In experimental series II a few carpospores survived or developed into few-celled sporelings at this temperature. These sporelings were characterized by thick walled cells and were poorly attached to the culture slide.

The relationship of growth rate to temperatures between these high and low extremes is presented in Figure 2A. The curve ascends to an optimum value

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FIGURE 1. Growth curves for germlings of *Chondrus crispus* at specified conditions of light and temperature. Germlings initiated from carpospores (A) or tetraspores (B). Arrows indicate points at which erect shoots were initiated for those cultures which produced them.

(of those employed) at 21.1° C, then drops off sharply. It is similar for sporelings derived from tetraspores and carpospores, although carpospore-sporelings regularly exhibited more rapid growth. For both types of sporelings the increase in growth rate per degree centigrade was greater between 4.4 and 11.7° C than between 11.7 and 21.1° C. Burns and Mathieson (1972) present results to the contrary; however, their experiments did not take into consideration the effect of a lag phase on growth.

At 23.9° C the growth rate was slightly less than that at 11.7° C. Furthermore, the sporelings grown at 23.9° C displayed morphological and cytological abnormalities. In these cultures some spores (whether tetraspore or carpospore) remained undivided, became highly vacuolated, and increased in size. This type of cell was also frequently observed in cultures at 26.7° C; it appeared incapable of cell division. Holdfasts that developed in cultures at 23.9° C were often irregular in outline and displayed an astral pattern of coloration. Color was confined to sectors extending from the center of the holdfast to the periphery. In contrast, holdfasts grown at 21.1° C were usually circular in outline, and only the cells of the rim, the location of active cell division, were colorless (Prince and Kingsbury, 1973a). Number of plants measured (1401 at 21.1° C and 429 at 23.9° C) and constancy of results in experimental series III support the reality of a sharp break in the growth curve between these two temperatures.

Comparison of the effect of light intensity on growth was made with tetraspore sporelings at two temperatures and with carpospore sporelings at a single temperature (Fig. 2B). The two types of sporelings responded similarly. Three intensities were employed. In all cases, greater intensity produced greater growth, but the effect was nearly level at intensities of 0.35 m watts/cm^2 and above. Photoperiod displayed a definite effect where temperature and intensity were held constant (Fig. 1A and B). At optimum conditions of 21.1° C and 0.35 m watts/cm^2, the growth rate of tetraspore and carpospore sporelings declined 31% and 19% respectively, with a 38% reduction in light duration (10:*14* *vs.* 16:*8*). Photoperiod of 16:*8* is approximately equivalent to that of the summer solstice at the latitude of Plymouth.

At optimum temperatures, the death of a spore is readily discernible and takes place over a brief period of time. Appreciable mortality occurred in all cultures at all temperatures, and has been found by others (Burns and Mathieson, 1972). Above this background level, however, effect of elevated temperature on spore mortality was marked (Fig. 2C). Spore mortality was assessed in this representation as a function of total spores and sporelings in cultures grown at the three intermediate temperatures after four days. For each spore type and temperature condition, 600–1200 spores were evaluated. Mortality increased with increase in temperature from 17–26% at 11.7° C, to 84–94% at 23.9° C, (to 100% at 26.7° C from earlier results). Carpospores showed a lower mortality at all temperatures than did tetraspores. We hypothesized that incipient mortality might not become apparent at 11.7° C within four days because of the "refrigeration effect" of this low temperature. Therefore, carpospores were reexamined after 8 days in the cultures at 11.7° C. Carpospore mortality had increased from approximately 15% to 45% in the intervening 4 day period. Carpospores tend to remain intact longer after death than do tetraspores which apparently have thinner and more fragile cell walls. Although spore mortality may be considerable (to 60%, depending on circumstances) at temperatures up to and including 21.1° C, mortality is closely related to increasing temperatures above that point, and rapidly approaches 100%.

Brief shock treatments at elevated temperatures present similar results. Exposure of 30 seconds at 35° C yielded only 3.5% mortality. At 45° C the same treatment yielded 100% mortality. Results of shock treatments at other temperatures and durations are presented in Figure 2D. In each case, a range of appreciable mortality with large standard deviations (including controls) occurs up to a certain temperature, and then increases rapidly with temperature. The temperature at which "entrainment" of mortality occurs is distinctly higher for the briefer period of exposure.

Cultures from tetraspores and carpospores, 187 days old and bearing erect shoots averaging 7.0 mm and 9.1 mm, respectively, were transferred from 11.7° to 26.7° C and held at the elevated temperature with periodic renewal of media for 35 days. After 16 days erect shoots of the tetraspore culture had assumed a yellowish-red color while the carpospore culture had turned from maroon to brick red. After 35 days, 41 tetraspore-derived individuals were $\frac{1}{2}$–$\frac{3}{4}$ dead (white). Several others were entirely dead (erect shoot and holdfast). The population macroscopically was light red. In the parallel cultures of carpospore-derived individuals, none were entirely or mostly dead, and the color remained a brick red.

Discussion

Our results suggest that *Chondrus crispus* will benefit in increased growth from increased temperatures through 21.1° C at Plymouth. At temperatures above that, growth drops sharply, and abnormalities increase. Temperatures above 21.1° C also have a profoundly deleterious effect on reproduction by causing death of spores if prolonged. Above 30° C even brief exposures will result in spore mortality.

Nuclear thermal electric generators are generally designed to spread a heated effluent onto the surface of the receiving waters to maximize the release of heat to the atmosphere. The zone of maximum heat from a generating station can be expected to lie primarily above populations of *Chondrus*. Any warming of the waters, particularly in the summer, brought about by operation of a power plant should result in increased growth of *Chondrus*, epiphytic algal species (Prince and Kingsbury, 1973b), as well as other eurythermal species. Increased competition for available substrate, nutrients, and light energy should result. Excessive epiphytism of *Chondrus* decreases the commercial value of the crop. If climatological or operational excursions result in impaction of maximally heated effluent against the *Chondrus* beds, however, a disaster may result.

Summary

Chondrus crispus was cultured under conditions of light intensity, photoperiod, mineral nutrition, and temperature approaching or slightly exceeding those to be expected for a subtidal population at Plymouth. Of these, only temperature beyond a certain point brought reduced growth or mortality. The growth curve breaks sharply between 21.1° and 23.9° C, and morphological abnormalities appear.

Cultures do not grow at all at 26.° C. As temperatures are elevated above 21.1° C, healthy spores die in culture in increasing numbers. Healthy spores exhibit mortality approching 100% when exposed to temperatures above 35–40° C, even for periods as brief as one minute, as might be encountered during entrainment in a thermal plume.

LITERATURE CITED

Adey, W. H., 1970. The effects of light and temperature on the growth rates in boreal-subarctic crustose corallines. *J. Phycol.,* **6**: 269–276.

Burns, R. L., and A. C. Mathieson, 1972. Ecological studies of economic red algae. II. Culture studies of *Chondrus crispus* Stackhouse and *Gigartina stellata* (Stackhouse) Batters. *J. Exp. Mar. Biol. Ecol.,* **8**: 1–6.

Ehrke, G., 1931. Uber die Wirkung der Temperatur und des Lichtes auf die Atmung und Assimilation einiger Meers- und Süsswasseralgen. *Planta Arch. Wiss. Bot.,* **13**: 221–310.

Guillard, R. R. L., and J. H. Ryther, 1962. Studies on marine planktonic diatoms, I. *Cyclotella nana* Hustedt and *Detonula conferuacea* (Cleve.) Gran. *Can. J. Microbiol.,* **8**: 229–239.

Ignatiades, L., and T. J. Smayda, 1970. Autecological studies on the marine diatom *Rhizosolenia fragilissima* Bergon. I. The influence of light, temperature, and salinity. *J. Phycol.,* **6**: 332–338.

Iwasaki, H., 1961. The life cycle of *Porphyra tenera in vitro. Biol. Bull.,* **121**: 173–187.

Kanwisher, J. W., 1966. Photosynthesis and respiration in some seaweeds. Pages 407–420 in Barnes, H. Ed., *Some Contemporary Studies in Marine Science.* Allen, Unwin, Ltd., London.

Lewin, J. 1966. Silicon metabolism in diatoms. V. Germanium dioxide, a specific inhibitor of diatom growth. *Phycologia,* **6**: 1–12.

Mathieson, A. C., and R. L. Burns, 1971. Ecological studies of economic red algae. I. Photosynthesis and respiration of *Chondrus crispus* Stackhouse and *Gigartina stellata* (Stackhouse) Batters. *J. Exp. Mar. Biol. Ecol.,* **7**: 197–206.

Newell, R. C., and V. I. Pye, 1968. Seasonal variations in the effect of temperature on the respiration of certain intertidal algae. *J. Mar. Biol. Ass. U.K.,* **48**: 341–348.

Newton, L., V. J. Devonald, and N. R. Jones, 1957. Recherches sur *Chondrus crispus* et quelques-unes des ses distant variétés. *Colloq. Int. Cent. Nat. Rech. Sci.,* **81**: 121–139.

Ott, F. T., 1966. *A Selected Listing of Axenic Algal Cultures.* Marine Biological Laboratory, Woods Hole, Massachusetts, 45 pp.

Prince, J. S., and J. M. Kingsbury, 1973a. The ecology of *Chondrus crispus* at Plymouth, Massachusetts. I. Ontogeny, vegetative anatomy, reproduction, and life cycle. *Amer. J. Bot.,* in press.

Prince, J. S., and J. M. Kingsbury, 1973b. The ecology of *Chondrus crispus* at Plymouth, Massachusetts. II. Field studies. *Amer. J. Bot.,* in press.

Provasoli, L., 1963. Growing marine seaweeds. *Proc. 4th Int. Seaweed Symp.,* **4**(1961) : 9–17.

Ring, P. D., 1970. Developmental and ecophysiological studies of *Chondrus crispus. M.S. thesis, University of Maine, Orono,* 73 pp.

Reference: *Biol. Bull.*, **145**: 589–597. (December, 1973)

PROTEIN POLYMORPHISM OF THE HYBRIDIZING SEASTARS *ASTERIAS FORBESI* AND *ASTERIAS VULGARIS* AND IMPLICATIONS FOR THEIR EVOLUTION

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We wish to present an initial comparison of biochemical and morphological data for the naturally hybridizing seastars *Asterias forbesi* (Desor) and *A. vulgaris* Verrill. The similarity in morphology between these two extremely abundant New England seastars has been noted repeatedly in the past hundred years (Verrill, 1866; Coe, 1912 and Aldrich, 1956). Nonetheless, they cannot be regarded as typical "sibling species" since 7 prominent characters of the external phenotype are characteristic of each form (Table I). In sampling local populations, no single characteristic appears to be completely reliable. However, a comparison of the complete list of characteristics usually allows definite assignment of a specimen to one taxon or the other.

Occasionally specimens are encountered which are clear morphological intermediates between the two species. Anecdotal information on the existence of hybrids has been reported (Clark, 1904; Sanchez, in Edmondson, 1966, page 41). In laboratory cultures, eggs of each species are able to be fertilized by sperm of the other species (Ernst, 1967). Hybrid formation evidently does not lead to massive introgression since hybrids have been found only at localities where typical "good" representatives also occur. That there is some introgression is evidenced by the lack of complete reliability of any one morphological characteristic. The specific mechanism evidently blocking introgression is unknown. The claim has been made that "introgressed individuals are normally eliminated by natural selection" (Mayr, 1963, page 132), although limited experimental evidence suggests the opposite (Lewontin and Birch, 1966).

Although *Asterias forbesi* ranges from the Gulf of Mexico to Maine, (Fig. 1), from intertidal to 100 m depths, north of Cape Cod it chiefly occurs in warmer waters of inshore bays, and is increasingly rarer. Its place is taken by *A. vulgaris* which is found from Cape Hatteras to Labrador, and south of Cape Cod it is limited to the deeper, colder offshore waters, to 650 m depths. The two species are sympatric in offshore areas south of Cape Cod and in a few harbors north of the Cape.

MATERIALS AND METHODS

Specimens of *Asterias forbesi* were collected from Woods Hole, Massachusetts, on the rocks in front of the Marine Biological Laboratory. Specimens of *A. vulgaris* were obtained at the east end of the Cape Cod Canal at Cape Cod Bay (Fig. 1). After starving individuals for three to ten days after collection, the hepatopancreas

TABLE I

Morphological characteristics used to distinguish Asterias forbesi *and* A. vulgaris *(from Verrill, 1866; Coe, 1921; and Aldrich, 1956)*

Character	*Asterias forbesi*	*Asterias vulgaris*
Shape of ray	Slender base, blunt tip	Thick base, tapered tip
Endoskeleton	Firm, formed of interlocking plates	Flaccid, formed of narrow, bar-like plates with large inter-spaces
Abactinal surface of the ray	Arched	Flattened
Abactinal spines	Scattered	Concentrated into a single median row on each ray
Major pedicellaria of adambulacral spines	Broad, with rounded tip	Elongate with pointed tip
Color of madreporite	Pale orange to red-orange	Cream
Optical peduncle	Eye unstalked	Eye born on a fleshy stalk

was removed from one or more of the starfish arms of a single individual and placed in a 1 ml centrifuge tube for grinding with a plastic rod rotated in an electric eraser. After centrifugation at about 21,000 rpm for three minutes, 10–20 μl of supernatant was placed in one of 24 slots of a vertical acrylamide electrophoresis unit (Aardvark Industries; Lombard, Illinois). Electrophoresis (7.5% gel) was usually performed for 2½ hours at 400 V (30–140 mAmp). In addition to analyzing several individuals of each species of *Asterias* on a single gel, at least one individual of the sea stars *Leptasterias tenera* and *Henricia sanguinolenta* was analyzed for comparison in the same way on each gel for each enzyme system.

Thirty-three protein systems were tested for suitability of which 9 yielded distinct band patterns (Fig. 2). Data from 19 to 72 individuals (usually 24–36) were used to characterize each band pattern. Procedures for staining gels were those reported by Shaw and Prasad (1970) for sorbitol dehydrogenase (SDH), phosphoglucose isomerase (PGI), and hexokinase (HK); by Hubby and Lewontin (1966) for leucine amino peptidase (LAP, with the minor change that Fast Black K is added at the same time as the substrate), general protein (GP, using amino black and coomassie blue), and malate dehydrogenase (MDH, at pH 7.6, 50 mg NAD, 50 mg NBT, 150 mg sodium salt of malic acid); by Brewer (1970) for fructokinase (FK); by Gooch and Schopf (1970) for esterase (E), and tetrazolium oxidase (TO); and by Yang as cited in Selander, Smith, Yang, Johnson and Gentry (1971) for glutamate oxalate transaminase (GOT). "Nothing" kinase (NK) and "Nothing" dehydrogenase (ND) develop using tetrazolium in the absence of added substrate.

RESULTS

Data are given only for individuals whose external morphology appeared typical of *Asterias forbesi* or *A. vulgaris*. Protein bands in the 9 protein systems are attributable to 27 band systems in *A. forbesi* and 26 in *A. vulgaris*, tentatively

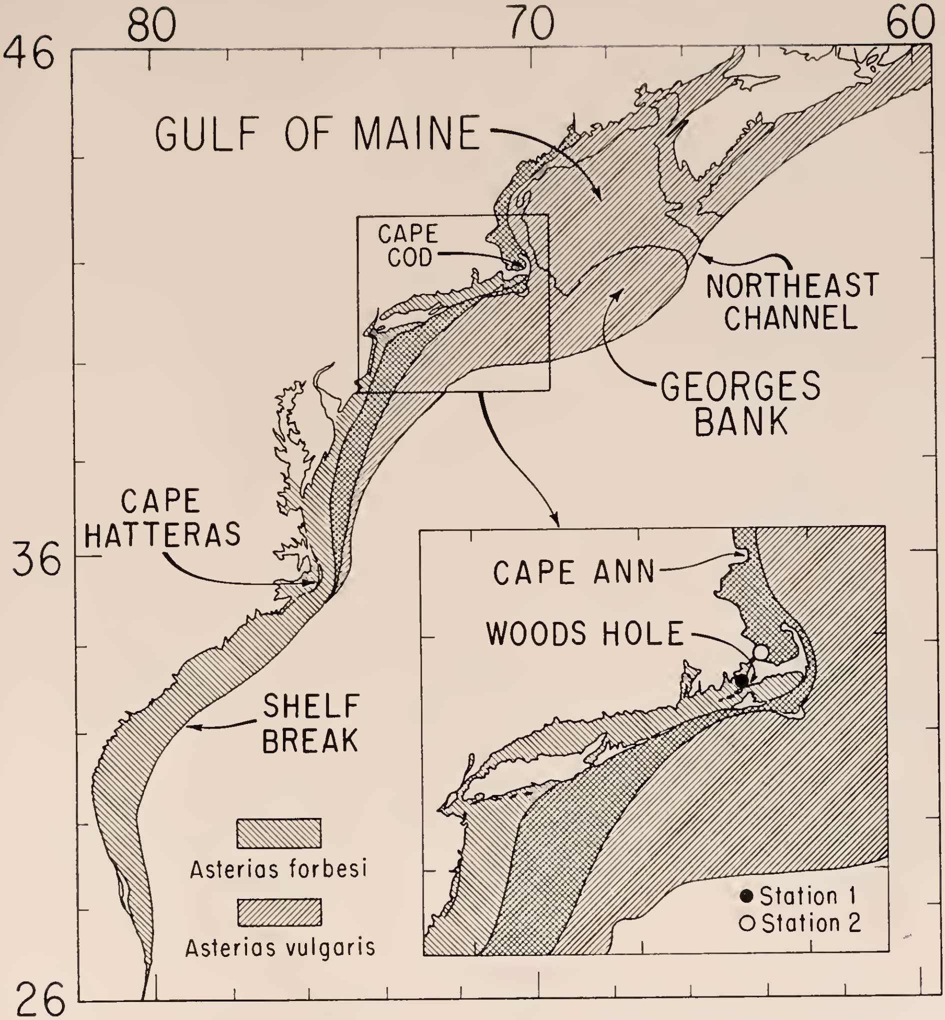
80
70
60
46
36
26
GULF OF MAINE
CAPE COD
NORTHEAST CHANNEL
GEORGES BANK
CAPE HATTERAS
SHELF BREAK
▨ Asterias forbesi
▨ Asterias vulgaris
CAPE ANN
WOODS HOLE
● Station 1
○ Station 2

FIGURE 1. Chart of Atlantic coast to show the distribution and probable overlap in range of the seastars *Asterias forbesi* and *A. vulgaris* (from Clark, 1904; Gray, Downey and Cerame-Vivas, 1968; and collections of L. S. Murphy); *A. forbesi* from station 1, and *A. vulgaris* from station 2.

equated with 27 and 26 gene loci. As is customarily done, we assume that the presumptive gene loci are a representative sample of structural loci in the genome of each species of *Asterias*. The biochemical data of this paper are restricted to a comparison of enzymes and other proteins of these two closely related species, although we note that in general the electrophoresis patterns of *Asterias* spp. were quite distinct from those recorded for *Henricia* and *Leptasterias*.

Allele frequencies for each locus (Table II) were considered according to the procedure of Nei (1972) to obtain an estimate of the degree of genetic similarity be-

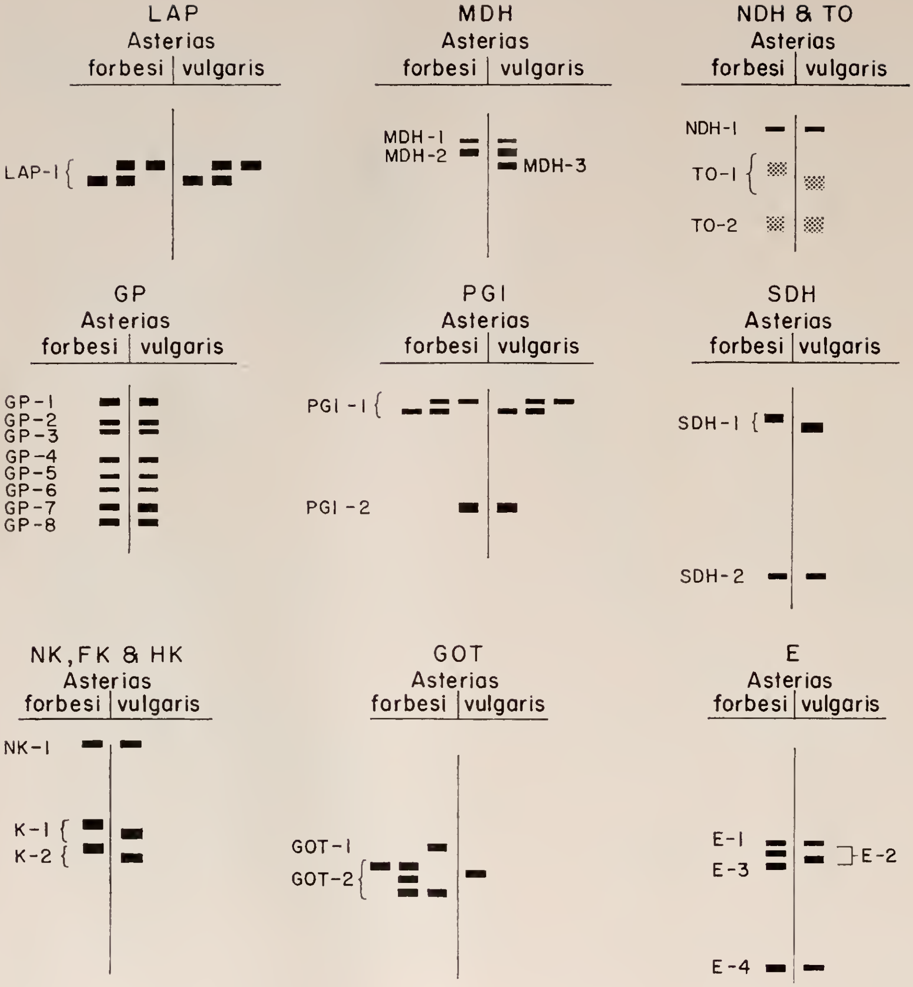
LAP
Asterias
forbesi
vulgaris
LAP-1
MDH
Asterias
forbesi
vulgaris
MDH-1
MDH-2
MDH-3
NDH & TO
Asterias
forbesi
vulgaris
NDH-1
TO-1
TO-2
GP
Asterias
forbesi
vulgaris
GP-1
GP-2
GP-3
GP-4
GP-5
GP-6
GP-7
GP-8
PGI
Asterias
forbesi
vulgaris
PGI-1
PGI-2
SDH
Asterias
forbesi
vulgaris
SDH-1
SDH-2
NK, FK & HK
Asterias
forbesi
vulgaris
NK-1
K-1
K-2
GOT
Asterias
forbesi
vulgaris
GOT-1
GOT-2
E
Asterias
forbesi
vulgaris
E-1
E-3
E-2
E-4

FIGURE 2. Diagram indicating band patterns and mobility relationships of protein zones of the seastars *Asterias forbesi* and *A. vulgaris*. For each protein the origin is at the top and the direction of mobility toward the bottom. Mobilities are given for typical runs of 2½ hours at 400 V and about 80 mAmp. Abbreviations stand for sorbitol dehydrogenase (SDH), malate dehydrogenase (MDH), tetrazolium oxidase (TO), general protein (GP), phosphoglucose isomerase (PGI), leucine amino peptidase (LAP), fructokinase (FK), hexokinase (HK), glutamate oxalate transaminase (GOT), Esterase (E), "nothing" dehydrogenase (NDH), and "nothing" kinase (NK). In addition to the systems indicated, future studies may reveal polymorphic systems between the origin and GP-1, and between GP-3 and GP-4. In PGI, a definite pink band develops just below PG-1. In SDH a narrow pink band occurs in a pinkish zone between SDH-1 and SDH-2. In MDH an uninterpretable but darkly staining band system exists below the TO-1 zone. MDH-1 is inhibited on gels to which has been added 50 mg HCN per 100 ml staining solution.

tween *Asterias forbesi* and *A. vulgaris*. On the average, each species shares 67% of its genes in common with the other species, for this sampling of gene loci. The genetic distance (D) is 0.397 which is the accumulated number of allelic differences per locus.

Table II

Allele frequencies for 27 loci in Asterias forbesi *and* A. vulgaris.
Abbreviations as in text. Band patterns in Figure 2

Locus	Allele	*Asterias forbesi*	*Asterias vulgaris*	Locus	Allele	*Asterias forbesi*	*Asterias vulgaris*
SDH-1	a	1.0	0.0	PGI-1	a	0.63	0.84
	b	0.0	1.0		b	0.37	0.16
GP-1	a	1.0	1.0	PGI-2	a	1.0	1.0
GP-2	a	1.0	1.0	GOT-1	a	1.0	0.0
GP-3	a	1.0	1.0	GOT-2	a	0.89	0.0
GP-4	a	1.0	1.0		b	0.0	1.0
GP-5	a	1.0	1.0		c	0.11	0.0
GP-6	a	1.0	1.0	LAP-1	a	0.66	0.79
GP-7	a	1.0	1.0		b	0.34	0.21
GP-8	a	1.0	1.0	E-1	a	1.0	1.0
NK-1	a	1.0	1.0	E-2	a	1.0	0.0
K-1	a	1.0	0.0		b	0.0	1.0
	b	0.0	1.0	E-3	a	1.0	0.0
K-2	a	1.0	0.0	E-4	a	1.0	1.0
	b	0.0	1.0				
MDH-1	a	1.0	1.0				
MDH-2	a	1.0	1.0				
MDH-3	a	0.0	1.0				
TO-1	a	1.0	0.0				
	b	0.0	1.0				
TO-2	a	1.0	1.0				
NDH-1	a	1.0	1.0				

Considering each species separately, for *Asterias forbesi* 11% of the loci (27) are polymorphic (at least 2 alleles with a frequency of more than 5%), and the heterozygosity per individual is 2.1% (calculated as in Lewontin and Hubby, 1966). For *A. vulgaris, 7.7%* of the loci (26) are polymorphic, and the heterozygosity per individual is 1.1%.

Discussion

A figure of 67% genic similarity in *Asterias forbesi* and *A. vulgaris* is within the range of values for studies of sibling species. In an investigation of nine triads of *Drosophila* (a sibling species pair plus one close relative), "As an overall average, members of a sibling pair have 50% of their proteins in common, while non-sibling members of a triad share only 18% of their proteins" (Hubby and Throckmorton, 1968, page 198). The percentage identity of proteins between sibling species in their study ranged from 22.5% to 85.7%. Comparing seastars and fruit flies, similar degrees of genetic differentiation result in quite different degrees of morphologic differentiation.

Other data comparing complexes of related species support the general conclusion that closely related species share a significant proportion of their loci in common. These include other species of *Drosophila* (Ayala, Mourão, Pérez-Salas, Richmond and Dobzhansky, 1970; Yang, Wheeler and Bock, 1972) and small mammals (Selander, Hunt and Yang, 1969; Johnson and Selander, 1971; Johnson, Selander, Smith and Kim, 1972). *Asterias forbesi* and *A. vulgaris* are interpreted as another example of "semispecies" (Mayr, 1963, page 118), that is, closely related species lacking complete reproductive isolation and sharing a significant proportion of their genes. This is the first case of semispecies in marine invertebrates for which this measure of genetic similarity is presented.

Of the many mechanisms which could result in reproductive isolation, the two which appear most likely are temperature tolerance and temporal isolation of spawning time. As charted 60 years ago, temperature now appears to be the limiting factor in the distribution of these species (Sumner, Osburn and Cole, 1913, page 113, charts 48, and 49). However, we are not aware of colaborative physiological studies of temperature tolerance on the reproduction or survival of these two species. Data exist on spawning time, but are inconsistent. Coe (1912) reported *A. forbesi* spawning in Long Island Sound in June. Loosanoff (1961) stated that spawning occurs in these same waters in July, but with local variation. *A. vulgaris* spawns in June and early July in Woods Hole (Field, 1892), and in late May and early June in the Gulf of St. Lawrence (Smith, 1940). All reports indicate a wide range of spawning times, with ripe inviduals found in the autumn.

How did *Asterias forbesi* and *A. vulgaris* originate? If glacial ice were to melt completely, sea level would rise about 70 m, submerging eastern Massachusetts (Emery, 1967a; 1967b). In addition, prior to the glaciations, glacial sediments, about 70 m on Georges Bank (Uchupi, 1968, 1970), would not have been deposited. Thus, at least in the early Pleistocene, species of *Asterias* and other shallow marine invertebrates would have freely ranged north and south of the present Cape. With subsequent sea level lowering, species ranges would have been split. At 15,000 years ago, sea level was approximately 130 m below its present level (Milliman and Emery, 1968; Emery, Niino and Sullivan, 1971). As sea level rose, arctic waters advanced into the Gulf of Maine, just as the sea today advances into fiords of Norway. Southern areas were receiving warm water from the Gulf Stream circulation. Perhaps 6000 to 7000 years ago some interbreeding would have been possible as the sill depth between the Gulf of Maine and the region to the south may have been breached. Presumably sufficient ecologic differentiation had occurred by then so that subsequent evolution has been largely independent. The alternative explanation—that *A forbesi* and *A. vulgaris* developed sympatrically—is of course possible. However, because of the reasonable presumed influence of glaciation in altering sea level and dividing species ranges, a sympatric origin seems to be less plausible, or at least to demand more stringent assumptions.

If the evolution of the northern *Asterias vulgaris* and the southern *A. forbesi* progressed as outlined above, then other pairs of species with similar origins are to be expected. E. L. Bousfield has recently (1973) published a detailed monograph of the amphipods of New England to depths of 30 m. According to our analysis of the distributional records in his monograph, 96 species have one boundary of their distribution associated with Cape Cod. We found 9 pairs of gemminate species, or approximately 20% of the amphipods of shallow waters. For other groups, Sumner, Osburn and Cole (1913) have a section entitled "Comparative Distributions of Closely Related Species" in which are cited records of possible equivalent types of species for hydroids, crabs, and clams. More recently documented examples may include mysids (Wigley and Burns, 1971), ostracods (Hazel, 1970), and ectoprocts (Osburn, 1933; Maturo, 1968; collections of T. J. M. Schopf).

These species pairs appear to be marine examples of the phenomenon well known on land of the influence of glaciation leading to geographic speciation, for which even the occurrence of hybrids is well established (Rand, 1948; Mayr, 1963, pages 369–372; Blair, 1951; Deevey, 1949, pages 1335–1338). Mayr (1963, page 372) states that "Most hybrid zones in the temperate region are the result of the fusion of populations expanding into the areas vacated by the retreating ice."

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SUMMARY

The seastars *Asterias forbesi* and *A. vulgaris* share 67% of their genes in common (based on 27 loci). These species are normally easily characterized by 7 prominent phenotypic differences but naturally occurring hybrids are found in localities with typical adults of the two species.

A. forebesi and *A. vulgaris* are thought to have evolved during the mid to late Pleistocene as a result of a restriction in the range of a more widely distributed Miocene or early Pleistocene form due to lowering of sea level and the coincident emergence of a disrupting land barrier (Cape Cod-Georges Bank). At least one local population of the ancestral species evolved into the present cold water form (*A. vulgaris*) during selection in an arctic-fed Gulf of Maine. Coincidently, at least one other local population of the ancestral species evolved with selection in warmer, southern waters into the present shallow water, temperate form (*A. forbesi*).

If *A. forbesi* and *A. vulgaris* have been derived from a late Tertiary wide-ranging species which underwent geographic partitioning during the late Pleistocene, then both their present distribution and their lack of total reproductive isolation are understandable.

LITERATURE CITED

✓ ALDRICH, A., 1956. A comparative study of the identification characters of *Asterias forbesi* and *A. vulgaris*. *Notulae Natur.*, **285**: 1–3.

AYALA, F. J., C. A. MOURÃO, S. PÉREZ-SALAS, R. RICHMOND, AND T. DOBZHANSKY, 1970. Enzyme variability in the *Drosophila willistoni* group, I. Genetic differentiation among sibling species. *Proc. Nat. Acad. Sci.*, **67**: 225–232.

BLAIR, W. F., 1951. Interbreeding of natural populations of vertebrates. *Amer. Natur.*, **85**: 9–30.

BOUSFIELD, E. L., 1973. *Shallow-water Gammaridean Amphipoda of New England.* Cornell University Press, Ithaca, New York, 312 pp.

BREWER, G. J., 1970. *An Introduction to Isozyme Techniques.* Academic Press, New York, 186 p.

CLARK, H. L., 1904. The echinoderms of the Woods Hole region. *Bull. U. S. Fish Comm. 1902,* **22**: 547–576.

✓ COE, W. R., 1912. Echinoderms of Connecticut. *Conn. State Geol. Natur. Hist. Surv.*, **19**: 1–152.

DEEVEY, E. S., JR., 1949. Biogeography of the Pleistocene. *Geol. Soc. Amer. Bull.*, **60**: 1315–1416.

EDMONDSON, W. T., 1966. *Marine Biology III.* New York Academy of Science Interdisciplinary Communications Program, New York Academy of Science, New York, 313 pp.

EMERY, K. O., 1967a. Some stages in the development of knowledge of the Atlantic coast of the United States. *Prog. Oceanogr.*, **4**: 307–322.

EMERY, K. O., 1967b. The Atlantic continental margin of the United States during the past 70 million years. *Geol. Ass. Can., Spec. Paper,* **4**: 53–70.

EMERY, K. O., H. NIINO AND B. SULLIVAN, 1971. Post-Pleistocene levels of the East China Sea. Pages 381–390 in K. K. Turekian, Ed., *The Late Cenozoic Glacial Ages.* Yale University Press, New Haven.

ERNST, E. J., 1967. The distribution, ecology, environmental behavior and possible hybridization of the sea stars *Asterias forbesi* (Desor) and *Asterias vulgaris* Verrill in the subtidal zone of Long Island. *Ph.D. dissertation, New York University* (University Microfilms #68–6060).

FIELD, G. W., 1892. The larvae of *Asterias vulgaris. Quart. J. Microscop. Sci. London,* **34**: 105–128.

GOOCH, J. L., AND T. J. M. SCHOPF, 1970. Population genetics of marine species of the Phylum Ectoprocta. *Biol. Bull.*, **138**: 138–156.

GRAY, I. E., M. DOWNEY AND M. J. CERAME-VIVAS, 1968. Sea-stars of North Carolina. *Fish. Bull.*, **67**: 127–163.

HAZEL, J. E., 1970. Atlantic continental shelf and slope of the United States—Ostracode Zoogeography in the Southern Nova Scotian and Northern Virginian faunal provinces. *U. S. Geol. Surv. Prof. Paper,* **529-E**: E1–E21.

HUBBY, J. L., AND R. C. LEWONTIN, 1966. A molecular approach to the study of genic heterozygosity in natural populations. I. The number of alleles at different loci in *Drosophila pseudoobscura. Genetics,* **54**: 577–594.

HUBBY, J. L., AND L. H. THROCKMORTON, 1968. Protein differences in *Drosophila.* IV. A study of sibling species. *Amer. Natur.*, **102**: 193–205.

JOHNSON, W. E., AND R. K. SELANDER, 1971. Protein variation and systematics in Kangaroo rats (*Genus Dipodomys*). *Syst. Zool.* **20**: 377–405.

JOHNSON, W. E., R. K. SELANDER, M. H. SMITH, AND Y. J. KIM, 1972. Biochemical genetics of sibling species of the Cotton Rat (*Sigmodon*). *Stud. Genet.*, **7**: 297–305.

LEWONTIN, R. C., AND L. C. BIRCH, 1966. Hybridization as a source of variation for adaptation to new environments. *Evolution,* **20**: 315–336.

LEWONTIN, R. C., AND J. L. HUBBY, 1966. A molecular approach to the study of genic heterozygosity in natural populations. II. Amount of variation and degree of heterozygosity in natural populations of *Drosophila pseudoobscura. Genetics,* **54**: 595–609.

LOOSANOFF, V. L., 1961. Biology and methods of controlling the starfish *Asterias forbesi* (Desor). *U. S. Bur. Commer. Fish., Fishery Leaflet,* **520**: 1–11.

MATURO, F. J. S., JR., 1968. The distributional pattern of the Bryozoa of the East Coast of the United States exclusive of New England. *Atti Soc. Ital. Sci. Natur. Mus. Civico Storia Natur. Milano,* **108**: 261–284.

MAYR, E., 1963. *Animal Species and Evolution.* Harvard University Press, Cambridge, Massachusetts, 797 pp.

MILLIMAN, J. D., AND K. O. EMERY, 1968. Sea levels during the past 35,000 years. *Science,* **162**: 1121–1123.

NEI, M., 1972. Genetic distance between populations. *Amer. Natur.*, **106**: 283–292.

OSBURN, R. C., 1933. Bryozoa of the Mount Desert Region. Pages 291–354 in W. Procter, Ed., *Biological Survey of the Mount Desert Region.* Wistar Institute of Anatomy and Biology, Philadelphia.

RAND A. L., 1948. Glaciation, an isolating factor in speciation. *Evolution,* **2**: 314-321.

SELANDER, R. K., W. G. HUNT, AND S. Y. YANG, 1969. Protein polymorphism and genic heterozygosity in two European subspecies of the house mouse. *Evolution,* **23**: 379–390.

SELANDER, R. K., M. H. SMITH, S. Y. YANG, W. E. JOHNSON, AND J. B. GENTRY, 1971. Biochemical polymorphism and systematics in the Genus *Peromyscus.* I. Variation in the old-field mouse (*Peromyscus polionotus*). *Stud. Genet.*, **6**: 49–90.

SHAW, C. R., AND R. PRASAD, 1970. Starch gel electrophoresis of enzymes—a compilation of recipes. *Biochem. Gen.* **4**: 297–320.

SMITH, G. F. M., 1940. Factors limiting distribution and size in the starfish. *J. Fish. Res. Board Can.*, **5**: 84–103.

SUMNER, F. B., R. C. OSBURN, AND L. G. COLE, 1913. A biological survey of the waters of Woods Hole and vicinity. *Bull. U. S. Fish. U. S.*, **31** (Section I): 1–544.

UCHUPI, E., 1968. Atlantic continental shelf and slope of the United States—Physiography. *U. S. Geol. Surv. Prof. Paper*, **529-C**: C1–C30.

UCHUPI, E., 1970. Atlantic continental shelf and slope of the United States—Shallow structure. *U. S. Geol. Surv. Prof. Paper*, **529-I**: I1–I44.

VERRILL, A. E., 1866. On the polyps and echinoderms of New England, with description of new species. *Proc. Boston Soc. Natur. Hist.*, **10**: 333–357.

WIGLEY, R. L. AND B. R. BURNS, 1971. Distribution and biology of Mysids (Crustacea, Mysidacea) from the Atlantic coast of the United States in the NMFS Woods Hole collection. *Fish. Bull.*, **69**: 717–746.

YANG, S. Y., I. L. WHEELER, AND I. R. BOCK, 1972. Isozyme variations and phylogenetic relationships in the *Drosophila bipectinata* species comples. *Stud. Genet.*, **7**: 213–227.

Reference: *Biol. Bull.*, **145**: 598-606. (December, 1973)

A NEW DESCRIPTION OF *SYNDESMIS DENDRASTRORUM* (PLATYHELMINTHES, TURBELLARIA), AN INTESTINAL RHABDOCOEL INHABITING THE SAND DOLLAR *DENDRASTER EXCENTRICUS*

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The genus *Syndesmis* was erected by Silliman (1881) for a rhabdocoel inhabiting the sea urchin *Echinus sphaera*. Describing a rhabdocoel in the urchin *Strongylocentrotus franciscanus*, Lehman (1946) defined yet another genus, naming the particular species *Syndisyrinx franciscanus*. Marcus (1949) suggested that *Syndisyrinx* was synonymous with the *Syndesmis* of Silliman (1881) and Stunkard and Corliss (1951) concurred with this view in their revision of the family Umagillidae Wahl. In this revision three species of *Syndesmis* were recognized: *S. echinorum* inhabitng several European sea urchins; *S. franciscana* (Lehman, 1946); and *S. antillarum* inhabiting Diadema antillarum (which was originally reported but not named by Powers in 1936). Also presented was a description of a fourth species, *S. dendrastrorum*, a newly discovered rhabdocoel inhabiting the sand dollar *Dendraster excentricus* at La Jolla, California.

Knowledge of the genus *Syndesmis* Silliman, 1881, was extended by Jennings and Mettrick (1968) who undertook nutritional and chemical studies on *Syndesmis franciscana* occurring in the Jamaican echinoid, *Lytechinus variegatus*. From similar studies on *S. franciscana* occurring in *Strongylocentrotus franciscanus*, Mettrick (1969) concluded that the Caribbean syndesmid was probably *S. antillarum*. Failing to cite Mettrick (1969), Jones and Canton (1970) reported additional studies undertaken on the distribution of *S. franciscana* in *L. variegatus* in the Caribbean.

In the original description of *Syndesmis dendrastrorum* Stunkard and Corliss, 1951, many of the internal organs were not observed or described. In addition, it was not determined whether the worms were found in the intestine or the coelomic cavity. The illustration presented in their description is probably not representative, as it was based on but two specimens, one of which was contorted. The purpose of my work was to determine the location and occurrence of the worm in the host echinoid and to clarify the internal relationships of the reproductive organs. Also contained in this report is the first record and description of juvenile stages of *Syndesmis*.

Materials and Methods

Collection and maintenance of hosts

Specimens of *Dendraster excentricus* were collected off Shaw's Cove at Laguna Beach, California, during the months of November, December, January, February, and March of 1971–72. Specimens were obtained in twenty to thirty feet of water with the aid of scuba equipment. The sand dollars were immediately placed in a sea water aquarium after which they were maintained in a tank equipped with running sea water. No attempt was made to feed the organisms, however specimens were observed to feed on algae growing on the walls of the holding tank.

Examination of hosts for presence of worms

The location of the rhabdocoel in the host echinoid was determined by examining both fixed and live specimens of *Dendraster*. Sand dollars were fixed by placing them in 80 per cent alcohol for 24 hours. Sand dollars were dissected by carefully cutting out a disc of the aboral surface of the test, approximately 4.5 cm in diameter. This disc was carefully lifted up to reveal the internal organs.

The coelomic cavity was examined under a dissecting microscope for the presence of worms. Samples of coelomic fluid were also examined by flushing out the test with sea water by means of a pipette. These washings were collected and observed under both a dissecting and compound microscope. Examination of the intestine was accomplished by removing it from the organism and placing it in a petri dish of sea water. In the petri dish the lumen of the intestine was systematically examined with the aid of dissecting needles under a dissecting microscope. The location, number of worms found, and the diameter of the test (measured across the widest part on the oral surface) were recorded for each host examined. Live hosts were examined for the presence of worms within a week after collection.

Microtechnique

Following fixation in formal-alcohol (Humason, 1967), worms were prepared either as whole mounts (60 specimens) or for sectioning (25 specimens). Whole mounts were stained with Grenacher's Borax Carmine or Harris' Hematoxylin (Humason, 1967). For sectioning, specimens were embedded in Paraplast (Sherwood Medical Industries, Inc.) at 65° C, sectioned serially at 8 and 10 microns, and stained with Mallory's triple stain (Humason, 1967). Both whole mounts and sections were mounted with Histoclad (Clay-Adams, Inc.).

The slides were examined using brightfield or phase contrast illumination with a Zeiss photo-microscope. Live worms were examined using phase contrast, brightfield, and Nomarski interference contrast microscopy. Composite drawings were prepared from living material, whole mounts, sections, and enlarged photographs of representative preparations.

RESULTS AND DISCUSSION

Previous work (Stunkard and Corliss, 1951) left unanswered whether *S. dendrastrorum* occurs in the intestine or the coelomic cavity. The dissection of both the fixed and live hosts in this study, however, revealed that *Syndesmis dendrastrorum* occurs in the intestine. Worms were found to occur from the level of the esophagus throughout the intestine to the rectum. From the examination of 23 sand dollars, ranging from 5.8 cm to 7.5 cm in diameter, a range of from 0 to 24 worms per host was observed.

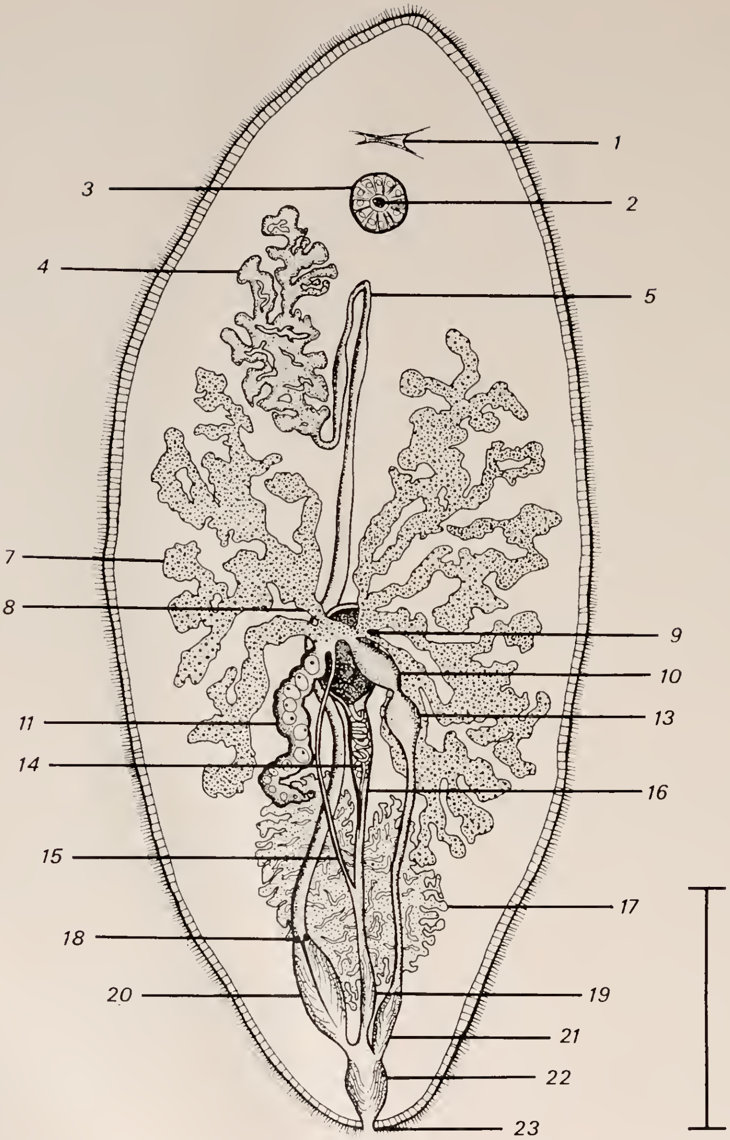

FIGURE 1. Dorsal view of *Syndesmis dendrastrorum* Stunkard and Corliss, 1951, with intestine omitted. Scale bar equals 200 microns; 1, brain; 2, buccal cavity; 3 pharynx; 4, testes; 5, sperm duct; 6 intestine; 7, vitellaria; 8, vitelline ducts; 9, egg capsule; 10, seminal receptacle; 11, ovary; 12, ova; 13, seminal bursa; 14, whip of egg capsule; 15, ovovitelline duct; 16, uterus; 17, cement glands; 18, penis; 19, female antrum; 20, male antrum; 21, bursal canal; 22, genital antrum; 23, genital pore.

As the earlier description (Stunkard and Corliss, 1951) was based on only two specimens, an incomplete account of the morphology was presented. A more detailed account follows, based on material collected at this new location. Living specimens of *Syndesmis* are opaque white, almost colorless, with extensive vitellaria and a large egg capsule. The worms are oval, slightly pointed at the anterior and posterior ends, widest laterally at the middle of the body; they are flattened dorsoventrally, with the dorsal side slightly convex (Figs. 1 and 2). Measurements of 25 mature specimens ranged from 0.52 mm to 1.23 mm in length; the width varied from 0.26 mm to 0.66 mm. Thickness measured about 30 microns at the edges to 90 microns at the thickest point.

The entire surface of the worm is covered with a ciliated epithelium. The cilia average 6 microns in length, compared to 3 microns as reported earlier (Stunkard and Corliss, 1951). The epithelial cells are about 6 microns in thickness and are irregular in shape.

The musculature and parenchyma is essentially the same as reported by Stunkard and Corliss (1951), and as reported for *Syndesmis franciscana* (Lehman, 1946). The epithelial cells are bounded inside by a basement membrane, under which lies a layer of circular, epithelial muscles. Under the circular muscles lie separated longitudinal fibers which occasionally transverse the parenchyma and are attached to the internal organs. The parenchyma, as in most Platyhelminthes, fills the spaces between the internal organs and the subepithelial muscles. It consists of large, irregularly shaped, vacuolated cells.

The brain is typical of previously described species. It is located approximately one-third of the way between the pharynx and the anterior end (Figs. 1 and 2). It consists of a nerve commissure connecting two ganglia.

An opening in the ventral epithelium approximately one-sixth of the distance from the anterior end of the worm forms the mouth. Immediately dorsal to the mouth is a buccal cavity surrounded by cells continuous with the epithelium. Connected dorsally to the buccal cavity is a doliiform pharynx, characteristic of the family Umagillidae, which averages approximately 54 microns dorsoventrally and 56 microns at its widest point (Figs. 1 and 2). The musculature of the pharynx follows that of *S. franciscana* (Lehman, 1946). The lumen of the pharynx is encircled by layers of distinctly staining vertical and circular muscle fibers. Radial fibers, extending from the lumen to the periphery of the pharynx, are also present with darkly staining cells filling the spaces between them (Figs. 1 and 2).

The esophagus opens into the intestine and extends dorsally from the lumen of the pharynx. As reported by Stunkard and Corliss (1951), the intestine lies directly under the dorsal epidermis, is medial in its position and extends from the pharynx to the level of the egg capsule (Fig. 2). The intestine is narrow and tubular, and is approximately 30 microns at its widest point. It consists of large, irregularly shaped, lightly staining cells. Food vacuoles or ingested material were not observed.

Paired, dendritic vitellaria fill most of the ventrolateral spaces in the middle one-half of the body. These structures extend anteriorly to the level of the testes and posteriorly to the level of the cement glands (Fig. 1). In the distal ends of the branches are primordial cells. These primordia give rise to amber colored yolk cells which travel toward the midline and the collecting ducts. The yolk cells pass from each side into 2–5 collecting ducts which unite at the midline immediately dorsal to the egg capsule at the mouth of the ovovitelline duct.

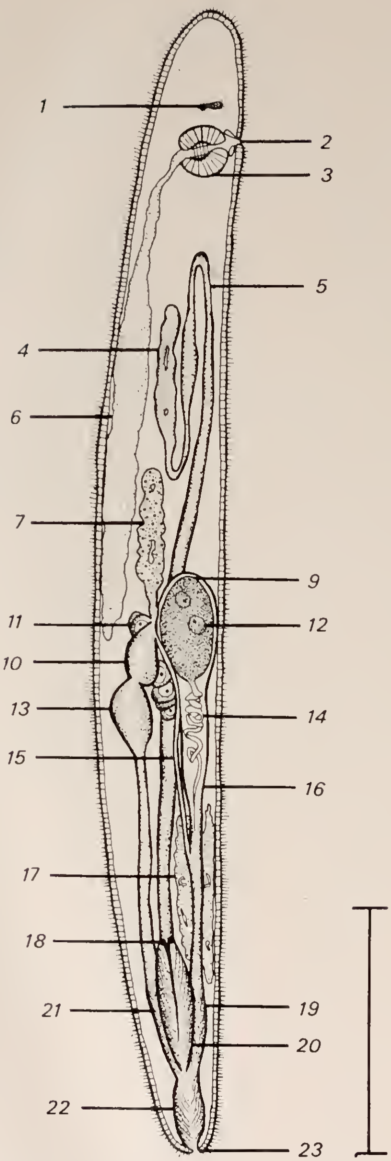

FIGURE 2. Sagittal section of *S. dendrastrorum.* Semidiagrammatic composite drawing. Scale bar equals 200 microns. Legend the same as in Figure 1.

The ovaries are single branches that arise at the mouth of the ovovitelline duct and extend posterolateral to the region of the cement glands (Fig. 1). The distal ends of the ovaries are sometimes lobed. Ovaries occur singly and were only seen to occur laterally in a cis relationship to a single testes. Stunkard and Corliss (1951) reported the occurrence of paired ovaries from the two specimens they examined. From my work it is clear that the existence of paired ovaries is not the usual case, as only 3 out of 25 specimens examined exhibited this condition. An ovary branch consists of a chain of ova, which are proliferated from primordial germ cells at the distal end. Mature ova are approximately 16 microns in diameter. The nuclei of these cells measure approximately 2 microns in diameter.

For inclusion in the genus *Syndesmis* Silliman, 1881, a specimen must possess paired ovaries (Stunkard and Corliss, 1951). Based on my findings, *S. dendrastrorum* must be considered an exception. While *S. dendrastrorum* has been seen to occasionally possess paired ovaries, these clearly represent minority cases. When paired ovaries have been observed, the animals also possess paired testes.

The presence of the large egg capsule in mature worms obscures and distorts the reproductive organs in many of the preparations thus making accurate observation of these structures difficult. Such a difficulty was encountered by Stunkard and Corliss (1951), which resulted in their omission of any detailed study of the reproductive organs. In addition to encountering distortion from the egg capsule in this study, I found that the ducts of the genital antrum were crowded together in such a way that they were difficult to resolve. The density of the stain that accumulated in the cement glands also hampered observation. Fortunately, several of the sections prepared in this study were exceptionally fine, enabling accurate observations upon which is based the following account.

The seminal receptacle is oval, about 22 microns at its widest point, and arises at the mouth of the ovovitteline duct (Figs. 1 and 2). Its presence is obscured in whole mounts by the position of the large egg capsule. Immediately posterior and confluent to the seminal receptacle is the seminal bursa, which measures approximately 20 microns across (Figs. 1 and 2). Arising out of the posterior end of the seminal bursa is the bursal canal, a tubular structure about 26 microns long and 10 microns in diameter. It proceeds posteriorly to the posterior limit of the cement glands, where it becomes widened and possesses cilia-like projections about 6 microns long extending from the walls into the lumen (Figs. 1 and 2). The posterior end of the bursal canal then narrows again, opening dorsally into the anterior end of the common genital antrum.

The common genital antrum is an oval structure which measures about 26 microns in diameter and possesses cilia about 10 microns long that extend into its lumen. While possessing the bursal canal as a dorsal-anterior extension, the common genital antrum is also joined laterally at its anterior end by the male antrum, and medially by the female antrum (Figs. 1 and 2). The common genital pore opens ventrally at the posterior end of the worm.

The female antrum is ovoid, and measures about 12 microns in diameter. Like the bursal canal, it also possesses cilia that extend posteriorly into its lumen. The female antrum extends anteriorly and gives rise to the uterus. In the region of the cement glands the uterus receives the ovovitelline duct as a dorsal diverticulum (Figs. 1 and 2). From this junction the ovovitelline duct extends anteriorly along the midline to the union of the vitellaria, ovaries, and seminal receptacle. The ovovitelline duct is approximately 6 microns wide and is capable of expansion to allow yolk cells from the vitellaria and ova to pass into the uterus. This arrangement is typical of the family Umagillidae.

A criterion for inclusion in the subfamily Umagillinae is that the "ovovitelline duct enters common genital antrum ventral or anterior to the common sperm duct" (Stunkard and Corliss, 1951, page 331). I have found that *Syndesmis dendrastrorum* does not exhibit this characteristic. On the contrary, in *S. dendrastrorum* the ovovitelline duct enters the uterus anterior to the female antrum. *Syndesmis franciscana,* another member of the subfamily, also does not meet these requirements. Lehman (1946) reports that the ovovitelline duct enters the anterior end of the female antrum.

The uterus extends anteriorly along the ventral surface to the middle of the body. At this point the anterior end of the uterus is enlarged and encloses the oval, amber-colored egg capsule (Figs. 1 and 2). The cuticularized egg capsule encloses a mass of yolk cells and one to four ova. In mature worms this egg capsule averages 98 by 55 microns. The posterior end of the capsule is elongated and narrowed, forming a whip. This structure is approximately 10 microns in diameter, and is coiled in the region of the uterus just posterior to the egg capsule (Figs. 1 and 2). The cuticularized egg capsule and whip stain red with Mallory's triple stain and Harris' hematoxylin.

The lobed structures which lie in the ventrolateral spaces of the body on each side of the uterus consitute the cement glands (Fig. 1). Small ducts from these glands enter the uterus and extend from the enlarged portion which contains the whip, posteriorly to the junction of the female antrum. It is probable that secretions from these glands are used in fastening the egg capsule to the substrate when it is expelled (Lehman, 1946).

Lying in the anterior region of the body between the pharynx and the vitellaria are the testes (Figs. 1 and 2). In mature worms these male structures measure on the average 195 microns long and 93 microns wide. The testes consist of closely packed lobes which communicate with the sperm duct through many smaller ducts as in *S. franciscana* (Lehman, 1946). These small ducts unite in an enlarged portion of the sperm duct. The sperm duct continues anteriorly towards the pharynx doubling back on itself about two-thirds of the way between the egg capsule and the pharynx. Continuing posteriorly, the sperm duct coils around the egg capsule through the parenchyma uniting with the base of the penis in the posterior region of the cement glands (Figs. 1 and 2). The sperm duct is approximately 24 microns in diameter and contains many longitudinal and circular muscle fibers. The testes occur singly and in a lateral cis relationship to the single ovary. Although possession of paired testes is a criteria for inclusion in the subfamily Umagillinae as set forth by Stunkard and Corliss (1951), exclusion from the subfamily is unnecessary, as a number of *S. dendrastrorum* have been observed to possess two of these organs.

The penis is a cuticular tube approximately 74 microns long and 2 microns thick. The base of the penis is a cuticularized collar which unites the sperm duct with the male antrum (Figs. 1 and 2). These cuticularized structures, like the egg capsule, also stain red with Mallory's triple stain and Harris' hematoxylin. The penis is sheathed by a large male antrum, measuring 30 microns in diameter at its widest point. The male antrum extends from the cuticular collar of the penis past the tip of the copulatory organ, entering the common genital antrum slightly laterally (Figs. 1 and 2). The walls of the lumen of the male antrum are heavily ciliated. Individual cilia measure about 20 microns long. Longitudinal muscle fibers in the walls of the male antrum and the sperm duct unite with the cuticularized collar of the penis. These fibers function as protractors and retractors of the penis as reported for *S. franciscana* (Lehman, 1946).

When my description and illustrations are compared with those originally presented by Stunkard and Corliss (1951), it might be inferred that different species are being described. However, the organisms described inhabit the same host, and the location and form of the vitellaria, the egg capsule and some of the other distinguishing characteristics presented in the two descriptions are similar. In addition, I have examined the type specimen deposited by Stunkard and Corliss, and have concluded that the differences are attributable to their contorted specimen.

During the dissection and examination of the hosts, I occasionally encountered small ciliated organisms. Closer examination of these forms revealed them to be juvenile stages of *S. dendrastrorum.* This is the first known account of juvenile stages in the entire family Umagillidae. The juveniles of *S. dendrastrorum* are opaque white, possessing the same shape as the adults. They vary from 0.28 mm to 0.77 mm in length and from 0.17 mm to 0.28 mm in width, depending on the stage of development. The youngest specimens, as judged by their small size, possess only a digestive system. In these young organisms a doliiform pharynx is present which occurs posteriorly, one-fourth of the distance from the anterior end. Unlike the adults, the intestine of the juvenile stages is clearly visible and quite large, filling most of the body posterior to the pharynx. As growth continues, the intestine of the worms becomes less prominent, probably as a result of differential growth, and occupies a smaller portion of the body. The vitellaria develops from a group of cells in the middle of the body. These structures, the first visible sex organs, branch laterally both in an anterior and posterior direction. By the time they have reached one-half their full size, ducts of the reproductive system appear in the posterior portion of the body. Following the development of the vitellaria, the testes, ovaries, and cement glands appear and the organism grows to become a mature adult. A preparation of several of these juvenile stages along with specimens of several representative adults has been deposited in the National Parasite Collection, United States Department of Agriculture, Beltsville, Maryland (Collection No. 72536).

From my work many exceptions to the criteria proposed by Stunkard and Corliss (1951) for the subfamily Umagillinae have become apparent. On this basis, I feel that further revision of the subfamily is warranted. In addition, there is no published evidence that members of the Umagillidae are parasitic *sensu stricto;* my observations on *S. dendrastrorum* indicate that these organisms are probably innocuous endocommensals.

I should like to thank Dr. Rosevelt L. Pardy, who guided me throughout this study.

SUMMARY

1. *Syndesmis dendrastrorum* occurs in the intestine of the sand dollar *Dendraster excentricus.*

2. A detailed redescription of *S. dendrastrorum* is presented, clarifying the internal relationships of the reproductive organs.

3. The most distinguishing characteristics of this species include single ovaries and testes occurring in a cis relationship, and a large male antrum.

4. Juvenile stages of *S. dendrastrorum* possess only a digestive system and lack reproductive organs.

LITERATURE CITED

Humason, G. L., 1967. *Animal Tissue Techniques.* W. H. Freeman and Co., San Francisco, 569 pp.

Jennings, J. B., and D. F. Mettick, 1968. Observations on the ecology, morphology, and nutrition of the rhabdocoel turbellarian *Syndesmis franciscana* (Lehman, 1946) in Jamaica. *Carib. J. Sci.*, **8** (1–2) : 57–69.

Jones, I., and C. E. Canton, 1970. Additional observations on the distribution of *Syndesmis franciscana* in the Caribbean. *Carib. J. Sci.*, **10** (1–2) : 71–72.

Lehman, H. E., 1946. A histological study of *Syndisyrinx franciscanus,* gen. et sp. nov., an endoparasitic rhabdocoel of the sea urchin, *Strongylocentrotus franciscanus. Biol. Bull.*, **91** : 295–311.

Marcus, E., 1949. Turbellaria Brasileiros (7). *Biol. Fac. Filos., Cienc. Letras. Univ. Sao Paulo, XCIX, Ser. Zool.*, **14** : 7–156.

Mettrick, D. F., 1969. Nutrition and chemical composition of the rhabdocoel turbellarian *Syndesmis franciscana*, with notes on the taxonomy of *S. antillarum. J. Fish. Res. Board Can.*, **26** (10) : 2669–2679.

Powers, P. B. A., 1936. Studies on the ciliates of sea urchins. A general survey of the infestation occurring in the Tortugas echinoids. *Pap. Tortugas Lab. Carnegie Inst., Washington,* **29** : 295–326.

Silliman, W. A., 1881. Sur un nouveau type de Turbellaries. *C. R. Acad. Sci., Paris,* **93** : 1087–1089.

Stunkard, H. W., and J. O. Corliss, 1951. New species of *Syndesmis* and a revision of the family Umagillidae Wahl, 1910 (Turbellaria: Rhabdocoela). *Biol. Bull.,* **101** : 319–334.

Reference: *Biol. Bull.*, **145**: 607–626. (December, 1973)

OBSERVATIONS ON *TUBULOVESICULA PINGUIS* (LINTON, 1910) MANTER, 1947 AND ON SYSTEMATICS OF THE HEMIUROID TREMATODES[1]

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Linton (1940) described *Dinurus pinguis* n. sp. from a large number of marine fishes. The specific description and figures were based on material from *Menidia notata*, with additions from specimens taken from other fishes. *Menidia menidia notata* (Mitchill) was recognized formerly as a northern subspecies, but the subspecies is no longer recognized by leading authorities. Linton admitted that in earlier publications he had assigned these parasites to other, different species, *e.g.*, specimens from *Anguilla rostrata* were identified as *Distomum grandiporum* (1898, page 520), and others from *Roccus lineatus* were referred to *Distomum rufoviride* Rudolphi (1898, page 512). Linton (1901) identified as *Distomum tornatum* Rudolphi specimens from *Fundulus heteroclitus* (page 442), from *Menidia notata* (page 444), and from *Roccus lineatus* (page 455). To *Distomum tornatum* he (1905) assigned specimens from *Synodus foetens* (page 355), *Tylosurus marinus* (page 356) and *Menticirrhus americanus* (page 399), all taken at Beaufort, North Carolina. It is questionable whether or not all of these determinations pertain to the same species. Furthermore, it must be recognized that fishes feed on smaller fishes and parasites of ingested fishes may be found in the digestive tract of predators.

In his (1940) paper, Linton listed the following species as hosts of *Dinurus pinguis:* the American eel, *Anguilla rostrata;* silver hake, *Merluccius bilinearis;* silversides, *Menidia notata;* kingfish, *Menticirrhus saxatilis;* toadfish, *Opsanus tau;* summer flounder, *Paralichthys dentatus;* common gurnard, *Merulinus carolinus;* northern barracuda, *Sphyraena borealis;* and the lizardfish, *Synodus foetens.* The material on which the species *D. pinguis* was based consisted of an assemblage of small trematodes, collected over a period of forty years, and obviously included representatives of more than one species and probably of more than one genus. The specific description was general, indefinite, equivocal and could comprise members of different species or even genera.

Two new genera, *Stomachicola* and *Tubulovesicula*, were erected by Yamaguti (1934) and assigned to the subfamily Dinurinae Looss, 1907. *Stomachicola* was based on *S. muraenescocis* Yamaguti, 1934 from the stomach of *Muraenesox cinereus,* taken in the Inland Sea of Japan and the South China Sea. *Tubulovesicula* was based on *T. spari* Yamaguti, 1934, described on a single specimen from the stomach of *Sparus macrocephalus* taken in the Inland Sea. Included in the genus were two new species; *T. anguillae* from the stomach of *Anguilla japonica* and *T. muraenesocis* from *M. cinereus* taken in the Inland Sea; *T. angusticauda* (Nicoll, 1915) from *M. cinereus* taken in North Queensland and assigned originally to

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Ectenurus Looss, 1907; and *T. lindbergi* (Layman, 1950) from various fishes taken in Peter the Great Bay and assigned orginally to the genus *Lecithaster* Lühe, 1901. Manter (1947) discussed the genera of the family Hemiuridae Lühe, 1901 and page 348 compared *Dinurus* Looss, 1907 with other closely related genera: *Ectenurus* Looss, 1907; *Lecithocladium* Lühe, 1901; *Magnacetabulum* Yamaguti, 1934; *Tubulovesicula* Yamaguti, 1934; *Stomachicola* Yamaguti, 1934; and *Erilepturus* Woolcock, 1935. Manter (1947) listed the generic features of *Tubulovesicula* and stated, page 350 "Type species: *T. spari* Yamaguti, 1934. Other species: *T. anguillae* Yamaguti, 1934; *T. muraenesocis* Yamaguti, 1934; *T. californica* Park, 1936; *T. pseudorhombi* Yamaguti, 1938; *T. lindbergi* (Layman, 1930) Yamaguti, 1934; *T. nanaimoensis* (McFarlane, 1935) n. comb. (synonym: *Dinurus nanaimoensis* McFarlane, 1935) *T. pinguis* (Linton, 1940) n. comb. (synonym: *Dinurus pinguis* Linton, 1940); *T. angusticauda* (Nicoll, 1915) Yamaguti, 1934." Manter (1954) discussed the status of species in the genus *Tubulovesicula*. He observed extensive variations in morphology and size of eggs and suppressed *T. californica* Park, 1936; *T. pseudorhombi* Yamaguti, 1938; and *T. muraenesocis* as synonyms of *T. spari*. He gave a key to six recognized species of *Tubulovesicula*. Sogandares-Bernal (1959) redescribed *Tubulovesicula lindbergi* (Layman, 1930) from specimens from the stomach of an unidentified eel and *Synodus* sp. taken near the mouth of Chiman River, Pelado Island, Panama Bay, Panama. He declared that *T. spari* is indistinguishable from *T. lindbergi* and the latter species becomes type of the genus. Sogandares-Bernal recognized only four species, *T. lindbergi* (Layman, 1930); *T. angusticauda* (Nicoll, 1915); *T. magnacetabulum* Yamaguti, 1939; and *T. pinguis* (Linton, 1940). Although various authors have mentioned *Tubulovesicula pinguis,* there is no existing description of the species other than the confused and inadequate accounts of Linton.

Material and Observations

During the summer of 1972, examination of a large number of *Menidia menidia* has provided information on the development and adult morphology of *T. pinguis* and permits a redescription of the species. The study was carried on from June 1 to September 15 at the Marine Biological Laboratory in Woods Hole, Massachusetts. The worms were found, not in the stomach but in the body cavity, with the oral suckers attached to the liver or to one of the large intestinal blood vessels. They are blood-suckers and their digestive ceca were filled with blood of the host. This fact accounts for the reddish color of living worms. Occasionally, juvenile specimens were found in the tissues, but always free and never encysted. The incidence of infection varied from ten to forty per cent. The intensity was low, usually one or two worms in a host; the largest number found in a fish was eighteen.

The worms are appendiculate hemiurids, with the ecsoma (retractile tail-like portion of the body) smaller and typically shorter than the trunk or body proper. When the ecsoma is retracted, the posterior end of the body and excretory pore are withdrawn and appear deep within the body. When fully extended, the ecsoma may be as long as the rest of the body and in living worms may measure 3.00 mm in length. In fixed and stained specimens, it is shorter and more retracted.

In general morphology, the worms agree with the generic diagnosis of *Tubulovesicula* as given by Yamaguti (1934, 1958, 1971) but the esophagus bifurcates posteriorly to form two lateral pouches, all lined with cuticula and provided with powerful muscular walls. In living worms these structures are conspicuous and in constant movement, churning the contents back and forth in all three pockets. These lateral pouches are similar to the "stomachs" or "crops" of other hemiurid trematodes. In the generic diagnosis of *Hirudinella,* Yamaguti (1958, page 252) stated, "esophagus of moderate length; ceca forming "stomach" portion at commencement, often dilated posteriorly with dark ingesta probably due to blood of the host." In the diagnosis of *Tubulovesicula* Yamaguti (1934) did not mention the existence of "stomachs." In his Figure 117, of *T. muraenesocis,* the anterior ends of the ceca are somewhat distended, but the figures of other species included in the genus do not show enlargements. In the diagnosis of *Tubulovesicula,* Yamaguti (1934) reported the specimens from the stomachs of their hosts, but the worms in *M. menidia* are in the body cavity, not in the stomach. Also, Yamaguti did not mention the existence of "stomachs" in the digestive tract. Such lack of agreement, if indeed it exists between the specimens described by Yamaguti and those from *M. menidia,* might suggest generic difference, but since it is not correlated with other disagreements, the species, *pinguis,* is retained in *Tubulovesicula.* To provide a more complete description of the species, figures of developmental stages are presented. Measurements of any specimen depend on the degree of sexual maturity, the particular region and extent of muscular contraction, and the amount of pressure during fixation. The sizes of the suckers and pharynx are less variable than the soft parts and yield the most reliable data.

Specific diagnosis of T. pinguis

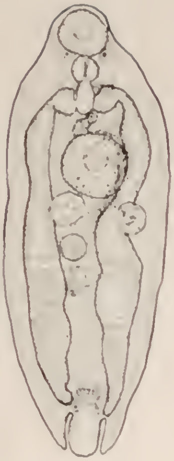
Gravid specimens are 3.80 to 6.50 mm long; 0.80 to 1.30 mm wide. The acetabulum, situated in the anterior third of the body, measures 0.38 to 0.48 mm in diameter. In fixed specimens the ecsoma is about one-fourth as long as the trunk.

Digestive system

The oral sucker is subterminal, 0.18 to 0.25 mm in diameter; the pharynx, adjacent to the oral sucker, is 0.13 to 0.16 mm in diameter. The esophagus is about as long as the pharynx; cervical glands discharge into its anterior portion and posteriorly it communicates with two lateral pouches, termed "stomachs," as reported earlier. The worms are hematophagus and the cervical glands presumably secrete an anticoagulant. The stomachs are slightly larger than the esophagus and measure about 0.16 mm in diameter. On either side, the stomach communicates by a short constricted portion with the cecum of that side. The ceca are lined with tall epithelium and extend the length of the body, terminating blindly near the posterior end.

Male reproductive system

The testes are spherical to oval to triangular, usually longer in the anteroposterior axis, 0.35 to 0.57 mm in diameter, situated immediately or a short distance posterior to the acetabulum. They may be opposite or either one may be slightly in advance. Sperm-ducts arise at their median faces and unite as they enter the posterior end of the seminal vesicle. The vesicle may be tubular, sinuous, or bent at an angle, or



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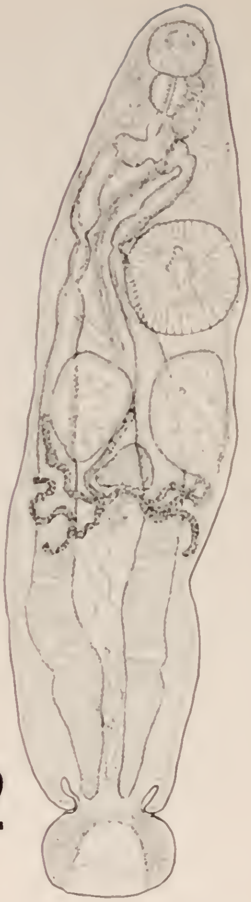

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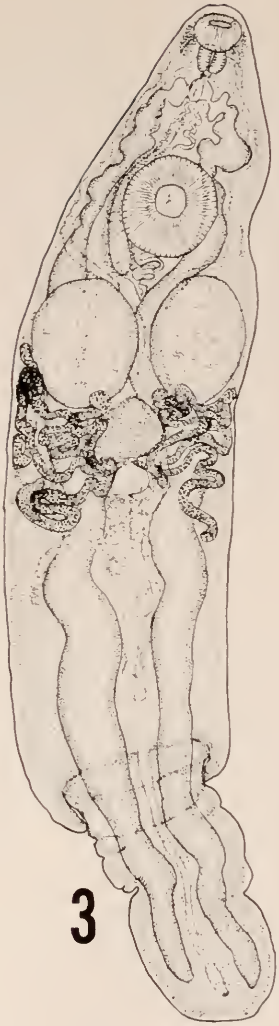
FIGURE 1. A dorsal view of a small, much flattened specimen, 1.00 mm in length. In it the ecsoma is retracted, the digestive and reproductive systems are clearly portrayed but the reproductive organs are rudimentary. The acetabulum is 0.15, the oral sucker, 0.10, and the pharynx 0.06 mm in diameter.

FIGURE 2. Ventral view of a specimen, 2.00 mm in length. In it the male system is just maturing. There are spermatozoa in the seminal vesicle but the female system is not yet functional. There are no ova and the cells in the vitelline tubules are small, without vitelline droplets in the cytoplasm. The acetabulum is 0.22, the oral sucker is 0.14, and the pharynx is 0.09 mm in diameter.

if filled with spermatozoa it may be saccate, 0.52 by 0.14 mm. The vesicle is anterodorsal to the testes; it is followed by a short, narrow duct that, posterior to the acetabulum, opens into a long, almost straight tube, encased in prostatic cells from its origin to about three-fourths of the distance from the acetabulum to the bifurcation of the digestive tract. Here it loses its covering of glandular cells, coils about for a short distance and joins the metratermal end of the uterus. The hermaphroditic duct enters a tubular to oval muscular sac that opens into the genital atrium. The positions and relations of the genital ducts vary with contractions of



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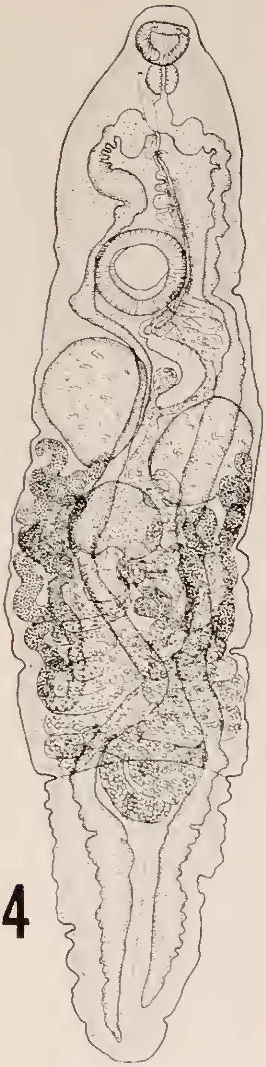

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Figure 3. Ventral view of a specimen 4.00 mm long. The ecsoma is 0.94 mm long. The worm is fully mature, with eggs in the initial portion of the uterus. In it, the uterine coils are not congested with eggs and other structures are visible. The acetabulum is 0.31, the oral sucker, 0.16, and the pharynx is 0.11 mm in diameter.

Figure 4. Dorsal view of a gravid specimen in which the anterior end is well extended and the reproductive ducts are conspicuous. In it the trunk is 3.30 mm long, 1.00 mm wide; the distance between the suckers is 0.65 mm. The ecsoma is 1.30 mm long, the acetabulum is 0.44, the oral sucker, 0.22, and the pharynx is 0.14 mm in diameter. The stomachs are 0.15 mm in diameter. The left testis is 0.50 by 0.47 mm; the right 0.50 by 0.38 mm; the ovary is 0.30 by 0.34 mm; the seminal vesicle is 0.50 by 0.14 mm; and the hermaphroditic muscular sac is 0.10 by 0.04 mm.

the body musculature and are seen best when the anterior region is extended. The genital atrium is narrow and is situated ventral to the pharynx.

Female reproductive system

The ovary is median to submedian, spherical to oval to reniform, often longer in the transverse axis, and 0.30 to 0.50 mm in diameter. The oviduct arises from the posterior face, receives a duct from the seminal receptacle, another from the vitelline reservoir, and enters Mehlis' gland, where it becomes the ootype, and from which the uterus takes origin. The seminal receptacle, which varies in size with the amount of sperm, is located posterior to the ovary, and the initial portion of the uterus may be filled with spermatozoa. The vitellaria consist of long tubular lobes, three on one side and four on the other, which discharge into the common reservoir. A short duct from the reservoir joins the oviduct immediately after the duct from the seminal receptacle. The arrangement of the vitelline lobes is not constant; the set of three may be on either side of the body. The uterus passes posteriad in close coils, often into the ecsoma, and then forward, between the testes, above or beside the acetabulum, and in the preacetabular area it is ventral to the male duct until their junction. Young eggs are thin-shelled, 0.020 by 0.013 mm in average measurement; eggs in the terminal portion of the uterus are thick-shelled, operculate, embryonated, and measure 0.018 by 0.012 mm. Eggs are slightly smaller in fixed and stained specimens.

Excretory system

The pore is almost terminal and the vesicle consists of a median stem which extends forward to the testicular level where it bifurcates and the arms pass anteriad and laterad in front of the testes and ventral to the digestive ceca. They continue a forward course to the level of the junction of the oral sucker and pharynx where they turn dorsad and mediad. At this point, each gives off a recurrent tubule and then they join above the posterior end of the oral sucker. This vesicular portion of the excretory system is filled with exceedingly fine globules. The fluid in the recurrent tubules is clear. These tubules pass posteriad, lateral or ventral to the digestive ceca, and receive branches formed by the union of capillaries that drain the flame-cells dispersed throughout the tissues. The body is thick, the parenchyma is dense, the pattern is complex, and details of the system have not been worked out. The only report of the flame-cell pattern in a mature hemiurid was made by Looss (1894) who found 2 [(11 × 32)] or 704 cells in *Azygia lucii*. The same pattern and cell number was found in the body of the cercaria of *Azygia sebago* by Stunkard (1956). Wootten (1957) found the same pattern and number of flame-cells in the body of *Azygia acuminata* and 2 [(5 × 32)] or 320 flame-cells in the tail of that species.

These trematodes from *M. menidia* are strikingly similar to members of the genus *Hirudinella* Garcin, 1730, studied by Nigrelli and Stunkard (1947). They agree in the sanguivorous habit, and the details of the digestive, excretory and reproductive systems. This morphological similarity implies close phylogenetic and systematic affinity. Yamaguti (1971) included Dinurinae Looss, 1906 as a subfamily in the Hemiuridae Lühe, 1901. Baer and Joyeux (1961) recognized Dinuridae Skrjabin and Gushanskaya, 1954 and Hirudinellidae Dollfus, 1932 as families in the superfamily Hemiuroidea.

Discussion

Tubulovesicula and Stomachicola

In a paper entitled, "The *Stomachicola rubea: Tubulovesicula pinguis* enigma," Sinclair, Smith and Sullivan (1972) reported *S. rubea* from twenty-eight species of marine fishes collected near Sapelo Island, Georgia, between 1 October, 1969 and the autumn of 1971. The species had been described by Linton (1910) as *Dinurus rubeus* n. sp. Manter (1931) had described *Dinurus magnus* as a new species from *Synodus foetens* taken at Beaufort, North Carolina and noted that it differed from *D. rubeus* in extent of the pars prostatica and seminal vesicle. Both species were transferred by Manter (1947) to the genus *Stomachicola* Yamaguti, 1934. The specific names, *rubeus* and *magnus* were incorrectly emended to *rubea* and *magna* respectively, apparently on the mistaken belief that *Stomachicola* is a feminine name. Yamaguti (1971) listed the species as *S. rubeus* and *S. magnus*.

Sinclair *et al.* (1972) suppressed *S. magna* (Manter, 1931) Manter, 1947 as a synonym of *S. rubea* (Linton, 1910) Manter, 1947. Furthermore, they predicated, (page 253) "an additional synonymy with *S. rubea* is the designation *Distomum tornatum* (in part) of Linton (1901, 1905, 1940), later referred to as *Dinurus pinguis* by Linton (1940) and Dawes (1940) and as *Tubulovesicula pinguis* by Manter (1947, 1954), Skrjabin and Guschanskaia (1954), Yamaguti (1958), Sogandares (1959) and Overstreet (1968)."

Although the life-history of the species is unknown, Sinclair *et al.* (1972) reported that *S. rubeus* uses a large number of small fishes as "transfer hosts." According to the report, these small fishes become infected in late spring or early summer; growth of the worms is continuous.; and they wander freely in the body cavity or tissues, often invading the liver, spleen, heart, kidney, swim bladder and somatic musculature. Such worms are able to attain full maturity, often leaving trails of eggs in tissues during passage. Response by their hosts eventually kills the worms which become enclosed in melanated cysts and "mummified" in late winter or early spring. Larger fishes become infected by eating "transfer hosts." The only fishes recognized as "true" definitive hosts were the eel, *Anguilla rostrata;* tarpon, *Megalops atlantica;* lizardfish, *Synodus foetens;* and the kingfish, *Menticirrhus americanus*. In these fishes, the worms, acquired by ingestion of transfer hosts, remain in the stomach. Accordingly, the authors regarded *T. pinguis* as merely a young form of *S. rubeus*. They noted that *M. americanus*, when small, was one of the more common transfer hosts, but carried *S. rubeus* in the stomach when it reached a larger size (about 30 cm). This dual site of infection in the same host species, depending on the size of the host, suggested that residence in a transfer host is a necessary part of the life-cycle of *S. rubeus*, since it is unlikely that the definitive hosts acquire the infection from the host (probably a small crustacean) that harbors the metacercaria. Tracing the growth of the parasite in *Synodus foetens*, they reported that in June, 32 worms had a total length of 0.96–4.96 mm (av. 2.52) with the ecsoma contributing 0–62 (av. 42%) of this length. By early September, 128 worms ranged in size from 1.28–9.55 mm (av. 5.99) with the ecsoma contributing 0–77 (av. 61%). By early October, 16 specimens had the following lengths: 3.0–9.7 (av. 8.24 mm); ecsoma 33–79 (av. 64%) of the total length. In *M. americanus* and *Cynoscion* spp., the worms attained a length of

9.44–14.40 mm by mid-winter and from the pattern of growth it would appear that the larger worms (22–25 mm) of this species could be more than one year of age. However there was no statement concerning the sites from which these specimens were taken, and since the same host species may serve as both transfer and definitive host, the data are of doubtful value.

Dinurus rubeus was described by Linton (1910) from *Lycodontis moringa* and *Lycodontis funebris* taken at Dry Tortugas, Florida. Specimens measured 22 mm in maximum length and 1.5 to 2.00 mm in greatest width. In a large specimen the oral sucker was 0.98, the acetabulum was 1.92, and the pharynx 0.42 mm in diameter. In Figure 151, of a total mount, the acetabulum is less than its diameter posterior to the oral sucker and the gonads are in the anterior one-fifth of the body length. *Dinurus magnus* Manter, 1931 was *Distoma tornatum* Linton, 1905 renamed. The specimens were from *Synodus foetus* and *Cynoscion nebulosus* taken at Beaufort, North Carolina. The sizes agreed with the measurements of *D. rubeus,* with the ecsoma about two-thirds of the total length. Manter (1931) distinguished *D. magnus* by a short pars prostatica and the extent of the seminal vesicle. As noted, Yamaguti (1934) erected the genera *Tubulovesicula* and *Stomachicola*. *Stomachicola* was based on *S. muraenescocis*, the type species. The worms are very long and slender, with the ecsoma some ten times as long as the trunk. The type specimen (Yamaguti, 1934, figures 105, 106) is 40.8 mm long and 1.89 mm wide. The largest immature specimen was 10.2 mm long and the largest mature one was 55 mm long. *Tubulovesicula* and *Stomachicola* were included in the subfamily Dinurinae Looss, 1907 but Yamaguti (1958) named *Stomachicola* type of a new subfamily Stomachicolinae in the family Hemiuridae. Yamaguti (1934) distinguished between *Stomachicola* and *Tubulovesicula* on the shape of the body, relative length of the ecsoma, and details in the shape of the seminal vesicle and extent of the pars prostatica. Manter (1947) transferred *D. rubeus* and *D. magnus* to *Stomachicola*. In *Tubulovesicula* the body is fusiform and the ecsoma, although variable in length, ordinarily is not more than one-half the total length.

The enigma posed by Sinclair *et al.* (1972) results from lack of information concerning the species under consideration. Unfortunately, the life-cycles of marine hemiurid trematodes are virtually unknown and evaluation of morphological data is dependent on knowledge of comparable features in other groups. The argument by Sinclair *et al.* that *S. magnus* is identical with *S. rubeus* is plausible but it is difficult to accept the identity of *T. pinguis* and *S. rubeus*. The idea that fully mature and gravid specimens of *T. pinguis,* four mm in length, if ingested by a larger host, can grow to a length of 20 mm or more and become stomachicolid in form, is contrary to accepted tenets of trematode development. It seems more probable that two closely related species may live simultaneously in the same host and that distinguishing features are not readily apparent. It is true that *T. pinguis* is primarily a parasite of the coelom and tissues but the worms must penetrate the wall of the digestive tract to enter the body cavity, and since only a few are present at any one time, they may be able to return. One distressing problem is to explain the fate of eggs shed by the worms in the closed coelom of *M. menidia*. If *T. pinguis* is merely a developmental stage of *S. rubeus,* the integrity of the genera is disrupted and the status of the several species assigned to the two genera becomes equivocal.

Systematic considerations

The trematodes from the stomachs of fishes constitute a large and diverse collection of genera, assigned by different authors to some twenty families in the superfamily Hemiuroidea Faust, 1929, the equivalent of the suborder Hemiurata Skrjabin and Gushanskaya, 1954. They have been known since ancient times and the name of the giant trematode from scombriform fishes, *Hirudinella marina* Garcin, 1730, is pre-Linnean. These worms are unique; they differ from other trematodes in structure and especially in the larval and developmental stages. In general, the body is strongly muscular, only slightly flattened, although in different groups, different regions of the body may be modified as adaptive responses to locations in their hosts and to subjective pressures. In certain species, the oral sucker may be retracted within the body and in one group, which includes *Tubulovesicula* and *Stomachicola,* designated as appendiculate, the posterior portion of the body may be retracted within the anterior portion. Looss (1907) discussed the morphology of these flukes and pointed out that the retracticle region is not a tail. He designated the anterior region as the soma and the retractile region as the abdomen. It was described as the ecsoma (appendix) by Nicoll (1915). Looss described the effect of contraction on the topography of the organs. The first comprehensive treatise on the systematics of the group was predicted by Odhner (1911) in the statement, page 528, "Mit der Erkenntnis dass alle Magendistomen bei Fischen in verwandschaftlichen Beziehungen auseinander stehen, sind wir wieder um ein gutes Stück nach vorwärts gegen das natürliche Digenensystem gekommen, und die eigentümlichen Hemiuriden haben durch ihre Ableitung von den Azygiiden einen morphologischen Hintergrund bekommen. Früher (1907) have ich schon die Didymozoiden von den Hemiuriden abgeleitet, eine Auffassung die sich seither bei mir nur erhärtet hat, und ich fuhre also in natürlichen System der Digenea die familien Azygiidae Odhn., Hemiuridae Lühe, und Didymozoidae Montic. in dieser Ordnung nacheinander auf."

The same taxonomic concept was espoused by Fuhrmann (1928). He observed, page 98, "Näher oder entfernter verwandt sind alle *Magen-Distomeen* der Fische, die eine grosse Entwicklungsreihe zu bilden scheinen, welche mit den Azygiidae beginnt und in der formenreichen (30 Genera), reich verzweigten Gruppe der Hemiuridae gipfelt." He noted that certain members of the group have migrated from the stomach, some to the intestine, some to the gall-bladder, some to the swim-bladder, while others have moved forward into the mouth and gills. From these considerations he concluded, "Aus solchen Formen sind dann wohl die eigentümlichen Zystenparasiten der Familie der Didymozoonidae hervorgegangen."

With increase in number of described species and genera, the families listed by Odhner have been elevated to higher taxonomic categories. They have been dismembered and new taxonomic units have been proposed. The classification of these trematodes has sustained numerous revisions and no system is universally accepted. The worms are chiefly parasitic in marine fishes but are encountered, though much more rarely, in migratory and freshwater fishes, and members of the family Halipegidae Poche, 1925 occur in amphibians. They have been reported from lizards and snakes but it is possible that these records are concerned with predation. The earlier arangements were based almost entirely on the morphology of adult worms, since data on life-cycles and larvae are meager and relatively recent.

Dollfus (1923) erected a superfamily Hemiurida, to contain the Hemiuridae Lühe, 1901, Accacoeliidae Looss, 1912, and Syncoeliidae Dollfus, 1923. Poche (1926) recognized the superfamily, in which he included five families: Hemiuridae Lühe, 1901; Azygiidae Odhner, 1911, and three new families Halipegidae, Isoparorchiidae, and Xenoperidae. Faust (1929) included the same families in a superfamily which he named Hemiuroidea. Markevich (1951) proposed the order, Hemiurata, but did not list the constituent families. Skrjabin and Gushanskaya (1954) demoted the Hemiurata to the rank of a suborder with a single superfamily, Hemiuroidea Faust. They accepted the five families of Poche and in the suborder included the following families: Accacoeliidae Looss, 1912; Hirudinellidae Dollfus, 1932; Syncoeliidae Dollfus, 1923; Haplosplanchnidae Poche, 1925; Coitocaecidae Ozaki, 1929; Bathycotylidae Dollfus, 1932; Sclerodistomatidae Dollfus, 1932, Ptychogonimidae Dollfus, 1936; and Derogenetidae Dollfus, 1950. In a revision, Skrjabin and Gushanskaya (1956) divided the Hemiurata into two superfamilies: the Hemiuroidea Faust, 1929 in which the hermaphroditic duct is free in the parenchyma, and the Azygioidea which have a true genital bursa. The latter superfamily contained the families Azygiidae Odhner, 1911; Hirudinellidae Dollfus, 1932; Xeroperidae Poche, 1925; and Liocercidae Skrjabin and Gushanskaya, 1956. In a further revision, Skrjabin and Gushanskaya (1960), the Hemiuroidea was reconstituted with fifteen families, including six new ones: Dinuridae Skrjabin and Gushanskaya, 1954; Lecithasteridae Skrjabin and Gushanskaya, 1954; Lampritrematidae Skrjabin and Gushanskaya, 1954; Elytrophallidae Skrjabin and Gushanskaya, 1954; and Aerobiotrematidae Yamaguti, 1958. Skrjabin and Gushanskaya (1960) did not recognize the Didymozoidae Poche, 1907 (Didymozoonidae Monticelli, 1888, renamed) as members of the Hemiurata.

LaRue (1957) proposed a new system of classification based primarily on life-history data and particularly on the development of the excretory bladder and associated structures. Two distinct superorders were recognized; The Anepitheliocystidia, in which the bladder remains membranous, and the Epitheliocystidia, in which the bladder is enclosed in mesodermal cells and its wall becomes epithelial. In his system, LaRue accepted the superfamily Azygoidea Skrjabin and Gushanskaya, 1956, which was elevated to the Azygiata, one of four suborders in the order Strigeatoidea (Anepitheliocystidia). The Azygiata contained two superfamilies: Azygioidea with two families, Azygiidae Odhner, 1911 and Bivesiculidae Yamaguti, 1939, and a new superfamily Transversotrematoidea with the lone family Transversotrematidae, Yamaguti, 1953. The Hemiurata Skrjabin and Gushanskaya, 1954 was included as one of two suborders in the order Opisthorchiida (Epitheliocystidia). It contained nine families: Hemiuridae, Halipegidae, Dinuridae, Lecithasteridae, Lecithochiridae, Bathycotylidae, Isoparochiidae, Ptychogonomidae, and Didymozoidae. The relegation of the families Azygiidae and Didymozoidae to different superorders was a striking departure from previous arrangements. Moreover, recent electron microscope studies by Krupa, Cousineau and Bal (1969) have confirmed the observations of Stunkard (1930), that the wall of the excretory bladder in *Cryptocotyle lingua* is syncytial and similar findings were reported for other species by Powell (1972).

Adopting a proposal advanced by Marie Lebour (1912) and supported in part by Sewell (1922), Dubois (1929) and Dollfus (1949), Odening (1960) erected a new system of classification. The Digenea were divided into two subclasses, Sporocystoinei and Redioinei, based on the type of larva produced in the primary host. In the former category, the cercariae develop in sporocysts; in the latter, they develop in rediae. In the scheme of Odening, the Hemiurata, Didymozoata Skrjabin and Sul'c, 1933, and Azygiata were included in the Redioinei.

Baer and Joyeux (1961) divided the class Trematoda into three subclasses: Aspidogastrea, Digenea, and a new subclass, Didymozoidea. In their treatment of the Digenea, they adopted the arrangement of LaRue (1957) for the Azygiata and the Hemiurata; twenty three families were included in the superfamily Hemiuroidea. The subclass Didymozoidea contained only the family Didymozoonidae Monticelli, 1888, with twenty-two genera. The removal of the Didymozoidae from the Digenea was based on their peculiar and aberrant morphology, on the development of gonochorism, and on a postulated direct development resulting from observations of Ishii (1935) and of Grabda (1947). The arrangement by Baer and Joyeux was analyzed and rejected by Stunkard (1963).

In a monograph on the trematodes of Brazil, Travassos, de Freitas, and Kohn (1969) revised the classification of the Digenea, changed the names of the orders by the use of a new ending, -formes, and described the species reported from Brazil. The Digenea contained thirteen orders and eighty-nine families. In the Hemiuriformes, they included nine families: Didymozoidae, Hemiuridae, Lecithochiriidae, Elytrophallidae, Isoparorchiidae, Dinuridae, and Mabiaramidae Freitas and Kohn, 1967. In the Didymozoidae, they listed only one species, *Unitubulotestis sardae* (MacCallum and MacCallum, 1916) Yamaguti, 1952 from the gills of *Sarda sarda*. The family Mabiaramidae contained a single species, *Mabiarama provesiculata* Freitas and Kohn, 1967, from the stomach of *Rachycentron canadus*.

The most recent contribution to systematics of the hemiurid trematodes, is in the monumental, *Synopsis of Digenetic Trematodes of Vertebrates,* by Yamaguti (1971). This is a revision and amplification of his volume I (1958) in the series, *Systema Helminthum.* As formerly, the parasites are arranged under primary divisions based on their hosts, but new information when available has been employed in the disposition of families. The classifications of the Digenea above the family level proposed by LaRue (1957), Odening (1960), and Travassos *et al.* (1969) were not accepted. Because information on life-histories of these flukes is so fragmentary, and for many groups entirely absent, Yamaguti accepted only a few superfamilies and in the majority of instances, no attempt was made to refer families to higher taxonomic categories. The Azygiidae, for example, are unplaced although three subfamilies are recognized. The superfamily Hemiuroidea is restricted to eight families: Hemiuridae, Mabiaramidae, Ptychogonimidae, Prosogonotrematidae, Bathycotylidae, Hirudinellidae, Lampritrematidae, and Sclerodistomidae. The Hemiuridae contains twenty-five subfamilies, many of them demoted from family status of other authors. The superfamily Didymozoidea (Monticelli, 1888) emend., is accepted with the single family Didymozoidae and twenty-four subfamilies. A new superfamily, Isoparochioidea was erected to contain seven families, whose species occur in the swimbladder rather than the stomach of fishes. The Accacoeliidae were removed from the Hemiuroidea, since their cercariae are not cystophorous, and assigned to a superfamily, Accacoelioidea, as suggested by Dollfus (1960a).

In the course of the past hundred years, a large number of trematodes have been described, many on inadequate and erroneous information and based often on a single specimen. New genera and higher taxonomic categories have been erected to receive these dubious species. Among the hemiurid species, lack of information on life-cycles and larval stages has limited descriptions to features of sexually mature specimens. Consequently, specific and generic diagnoses are often indefinite and uncertain. Indeed, Nasir and Diaz (1971) have suppressed *Synaptobothrium* v. Linstow, 1904; *Plerurus* Looss, 1907; *Sterrhurus* Looss, 1907; *Separogermiductus* Skrjabin and Gushanskaya, 1955; and *Magniscyphus* Reid, Coil and Kuntz, 1965 as synonyms of *Lecithochirium* Lühe, 1901.

Discussing phylogeny of the divers groups of Trematodes, Baer and Joyeux (1961), observed, page 657, "On a décrit jusqu' à aujourd 'hui plus de mille genres de Trématodes groupés en quelque cent familles don plusiers monotypiques. On peut cependant affimer que cette pléthore de genres ne correspond nullement à la réalité et que leur nombre sera certainement réduit dans une proportion notable lorsque les auteurs entreprendront une revision sérieuse. On constate trop souvent que des caractères spécifiques sont prétextes à créer des genres nouveaux, sans même que la limite morphologique de ceux-ci ait fait l'objet d'une investigation sérieuse. L'emploi abusif de clés dichotomiques trop simples, c'est à dire qui sont fondées principalement sur des caractères superficiels ou variables, conduit, inévitablement vers une situation inextricable dont la conséquence est de surcharger la litterature scientifique d'une nomenclature aussi encombrante qu'inutile. L'existence de près de trente families monotypiques accentue le malaise taxonomique que l'on ressent en face due système actual dont le nombre des sous-families monotypiques est également trop élevé, laissant l'impression que le choix des bases de la classification des trématodes est arbitraire."

The genetic and taxonomic relations of the hemiuroid trematodes are yet obscure. They comprise a homogeneous, monophyletic group of digenetic forms. Primarily parasitic in the stomachs of marine fishes, some of them have migrated to other organs, some have invaded freshwater hosts, and members of one family, the Halipegidae, infect amphibians. Classification is based almost entirely on the adult generation since knowledge of life-cycles and developmental stages is meager. Life-cycles have been elucidated for several halipegid species, but this is a peripheral area in which events are probably greatly modified. For marine species a few life-cycles have been pieced together, but only one, that of *Lecithaster confusus* Odhner, 1905, has been experimentally demonstrated (Hunninen and Cable, 1943). As their hosts evolved and occupied new environments, there have been corresponding changes in the morphology, physiology and development of their parasites. Differences of opinion on the significance of particular features have led to the formation of diverse taxonomic arrangements as noted above. Minor variations have been overemphasized with the unjustified multiplication of taxonomic units, as observed by Baer and Joyeux (1961). When adequate information on life-cycles and developmental stages becomes available, existing differences may be explicable, differences of opinion may be resolved, and an acceptable system of classification may be formulated.

Life-cycles and classification

The wide divergence of opinion concerning the systematics and classification of the hemiurid trematodes is the result, in large measure, of lack of knowledge of their life-cycles and developmental stages. Data are meager, fragmentary, often faulty, and sometimes erroneous. Observations extend back for more than a century, (*q.v.* Dollfus, 1923, 1950a, 1954, 1960b) and unencysted metacercariae have been found in tow-nettings, and in a variety of planktonic organisms, chiefly crustaceans, ctenophores and chaetognaths. Dollfus (1923) noted that it is difficult if not impossible to recognize the adult stages and identify the larvae. He listed references to twenty-four species that occur in marine invertebrates. In addition, he noted that encysted progenetic metacercariae had been reported from various teleost fishes. Since the worms were gravid, they had been identified and referred to *Lecithochirium rufoviride* (Rudolphi, 1819) Lühe, 1901, to *Lecithochirium gravidum* Looss, 1907, and to *Synaptobothrium caudiporum* (Rudolphi, 1819) v. Linstow, 1904. The three species were included in the subfamily Sterrhurinae. Dollfus (1950a) enumerated the five essential characters of the cystophorous cercariae and listed the hosts and geographic distribution of the designated species. Thirty-nine species were described from gastropod hosts and one, *Cercaria prenanti* Arvy, 1940, from the scaphopod, *Dentalium dalli* taken off the northwest coast of France. This species was reported by Ching (1960) from the Pacific coast of the United States, at Friday Harbor, Washington. Contrary to earlier statements, Dollfus (1950a) predicated that all cystophorous cercariae belong in the superfamily Hemiuroidea; that the group is homogeneous and contains only those species in which the cercariae are cystophorous.

In a supplemental report, Dollfus (1960b) published a list of the distomes of chaetognaths, including those assigned to the Hemiuroidea. He predicated that the chaetognaths ingest the worms with food, either directly or in copepods or other small planktonic invertebrates which harbor the larvae. Thus, they may be either secondary intermediate hosts or merely facultative and supplementary associates intercalated in the life-cycle.

Szidat, Angelescu and Siccardi (1950) identified mature worms from the abdominal cavity of *Clupea melanostoma* taken in the Rio de la Plata, Argentina, as *Dinurus breviductus* Looss, 1907. Some were alive, others dead, and the disintegrated remains of others were centers of melanistic capsules containing eggs. Other small immature specimens were in caseous nodules on the wall of the stomach of *Raphiodon* (= *Cynodon*) *vulpinus* from the same locality. The normal host of *Dinurus breviductus* is *Coryphaena* sp., and Szidat *et al.* (1950) considered *Clupea melanostoma,* a freshwater species, to be an ancillary intermediate host, a "segundo huesped auxilliar." Commenting on this report, Dollfus (1954) declared that the worms encysted in teleost fishes, that earlier he had regarded as progenetic metacercariae, were actually adults which had perished in an aberrant host and outside their normal habitat in the intestine. He observed that the "cysts" were not secreted by the parasite but were connective tissue capsules produced by the hosts as a reaction to invasion. He cited other examples of hemiurid worms that had become mature in abnormal situations. The account by Sinclair *et al.* (1972) recalls the earlier one by Szidat *et al.* and in both instances the parasites had been assigned to the genus *Dinurus.*

The position of the Didymozoidae is equivocal. They are tissue parasites of marine and rarely, of freshwater fishes. They occur in the mouth, gills, skin, body cavity, muscles and connective tissue. In part they are cyst-dwelling, often in pairs, with partial or complete separation of sexes. Some are monostomes, others are distomes, and the peculiarities of morphology are clearly adaptations to the tensions imposed by their locations in their hosts. Ishii (1935) reported direct development of *Didymocystis katsuwonicola*. The embryonated eggs measured 0.017 to 0.019 mm in length and 0.010 to 0.012 mm in width. Each contained a larva whose anterior end bore a sucker and a double crown of alternating large and small spines. The penetration of the larva into the branchial epithelium was not observed, but similar larvae, about 1.0 mm in length, were found under the epithelium and it was presumed that the miracidium had transformed into the juvenile. Grabda (1947) made similar observations on the life-cycle of *Nematobothrium sardae* at the Varna Laboratory on the Black Sea. He found the worms in pairs on the gills of fishes, and reported penetration of young worms into the tissue. However, he stated, page 175, "L'auteur est parvenu à examiner à quatre reprises, la pénétration des individus du parasite dans la branchie du poisson. Malheureusement ces observations n'ont été faites que la préparations fixées." The accounts of Ishii and Grabda present no evidence that the miracidium penetrates the epithelium of the gills of fishes and Yamaguti (1958) questioned the account of Ishii. Developmental stages that appear to be didymozoid cercariae have been discovered in marine snails and unencysted metacercariae in crustaceans and juveniles in the intestine of small fishes portend a digenetic life-cycle. The statement of Yamaguti (1971, page 334) apparently establishes a digenetic cycle, with developmental stages from those found in crustaceans to those developing in the final hosts.

Skrjabin and Gushanskaya (1954) observed that complete life-cycles of members of the Hemiurata, for the most part, are known only for species in freshwater hosts. They noted that this is not surprising since freshwater conditions facilitate experimentation. These freshwater species are members of the family Halipegidae. The group is unique since it occurs in both marine and freshwater fishes and in both caudate and anuran amphibian hosts. Life-cycles are known for members of three genera, *Halipegus*, *Azygia*, and *Proterometra*. In the genus *Halipegus*, life-cycles are known for four species. The type, *H. ovocaudatus* (Vulpian, 1858) Looss, 1899 occurs in the oral cavity and esophagus of European frogs. Leuckart (1886) obtained experimental infections of *Planorbis planorbis* by feeding eggs of the parasite. In collaboration with a student, Creutzburg, Leuckart (1889) observed the emergence of the miracidium from the egg, the development of the generations in the snail and the formation of the cercaria, identified as *Cercaria cystophora* Wagener, 1886. The descriptions of the experiments and results were included in the dissertation of Creutzburg (1890). Sinitzin (1905) reported that *Cercaria cystophora* from *Planorbis marginatus* is eaten by nymphs of the dragonfly, *Calopteryx virgo*, develops in the hemocoele, and is the larva of *H. ovocaudatus*.

The life-cycles of American species have been worked out for *Halipegus occidualis* Stafford, 1905 by Krull (1935); for *Halipegus eccentricus* Thomas, 1937 by Thomas (1939); and for *Halipegus amherstensis* Rankin, 1944 by Rankin (1944). Thomas reported that the young worms remain in the stomach of tadpoles until metamorphosis, when they migrate to the oral cavity and Eustachian tubes. In all of these studies, the cystophorous cercariae were ingested by copepods and developed as unencysted metacercariae in the hemocoele of the crustaceans. Metacercariae were found as natural infections in both dragonflies and damselflies but experimental infections of nymphs were not obtained. Macy, Cook and DeMott

(1960) found that in Oregon, the cercariae of *H. occidualis* are ingested by ostracods, *Capridopsis vidua* (O. F. Müller), which serve as second intermediate hosts in that area. They proposed the term, Cercariocyst, for the cercaria of halipegid species. They found natural infections in dragonflies but stated, (1960, page 15) "Clearly, dragonfly nymphs are unnecessary for the completion of the cycle and become infected simply because they happen to eat ostracods along with other food." Adult worms were recovered from *Taricha granulosa* that had been fed infected ostracods.

Knowledge of azygiid life-cycles date from the report of Szidat (1932) that *Cercaria mirabilis* Braun, 1891, from *Lymnaea palustris* develops in *Esox lucius* and is the larva of *Azygia lucii* (Müller, 1776) Lühe, 1909, a common parasite in the stomach of salmonid fishes, especially species of *Esox*, in Europe. Life-cycles of North American species have been worked out for *Azygia sebago* Ward, 1910 by Stunkard (1956); for *A. acuminata* Goldberger, 1911 by Wootton (1957); and for *A. longa* Leidy, 1851 by Sillman (1962).

Members of the azygiid genus *Proterometra* Horsfall, 1933 also occur in North American freshwater fishes. Life-cycles have been elucidated for seven species: *Proterometra macrostoma* (Faust, 1919) Horsfall, 1933 by Horsfall (1934) and by Dickerman (1934); *Proterometra catenaria* Smith, 1934 by Smith (1934); *Proterometra hogesiana* Smith, 1936, by Smith (1936); *P. sagittaria* Dickerman, 1946 by Dickerman (1946); *Proterometra dickermani* Anderson, 1962 by Anderson and Anderson (1963); *Proterometra albacauda* and *Proterometra septimae*, both described by Anderson and Anderson (1967) with life-cycles recorded in the same publication. Anderson and Anderson (1963) reported that *P. dickermani* from *Goniobasis livescens* completes the life-cycle in the snail host. Metacercariae become gravid in the mollusk; eggs were embryonated and miracidia were obtained. They may produce new infections in the same snail or, if eggs are ingested, other snails may become infected. No naturally infected fishes were found but experimental infections were produced in sunfishes, *Lepomis* spp. and bass, *Micropteroides salmoides*.

Stages in the life-history of other halipegid species are known at least in part. Dollfus (1950b) described *Halipegus africanus* n. sp. from *Rana mascariensis* taken in the Belgian Congo and mature cystophorous cercariae in rediae from *Biomphalaria katongae*. The life-cycle was based on morphological agreement, not on experimental infection. Tang (1951) described *Genarchopsis chinensis* n.sp., from freshwater fishes in China with an account of the life-cycle. The cystophorous cercariae developed in *Melania foretiana*. The species was named type of a new genus, *Tangiopsis*, by Skrjabin and Gushanskaya (1955). Yamaguti (1954) reported cystophorous cercariae from *Semisulcospira libertina* which, when fed to *Cyclops* spp., developed into metacercariae with the characteristic features of the genus *Genarchopsis* Osaki, 1925 (syn. *Progonus* Looss, 1899; *Genarches* Looss, 1902, both names preoccupied). The genus has species in marine and freshwater fishes in Japan, Russia and India.

Genarchella genarchella Travassos, Artigas and Pereira, 1928 was described from the esophagus and stomach of *Acestrorhamphus* sp. in Brazil. Szidat (1956) found the larval generations in *Littoridina australis*. According to his account, the cercariae do not leave the rediae but become sexually mature and produce eggs. When infected snails are eaten, the eggs are liberated and the metacercariae grow and again become gravid. He predicated that the old snails perish in late fall and winter and the rediae they contain, together with the gravid metacercariae, emerge into the water. Young specimens of *L. australis* become infected by eating the eggs with food. The miracidia emerge, develop into sporocysts in the liver and begin the formation of rediae.

A remarkable and anomalous situation obtains in the related genus *Bunocotyle* Odhner, 1928, which contains unusual parasites of freshwater fishes. The type species, *B. cingulata* Odhner, 1928 was described from *Perca fluviatilis* taken on the east coast of Greenland, from *Acerina cernus* of the Baltic, and from *Silurus glanis* of the Volga delta. The genus was designated type of the subfamily, Bunocotylinae Dollfus, 1950, which was included in the family Halipegidae by Skrjabin and Gushanskaya (1954) and in the family Hemiuridae by Yamaguti (1971). Chabaud and Biguet (1955) described cystophorous cercariae from *Hydrobia stagnalis* taken from a canal near the Étang-du-Canet (Pyrénées Orientales) and their development in copepods, *Poppella guernei,* to progenetic metacercariae that were identified as *Bunocotyle cingulata* Odhner, 1928. Markowski (1936) had described progenetic metacercariae from *Hydrobia ventricosa,* taken in the Baltic, as *Metorchis progenetica.* As many as fifty gravid worms were found in a single snail. Chabaud and Buttner (1959) showed that the larva described by Markowski (1936) is a species of *Bunocotyle* for which they proposed the name *Bunocotyle progenetica* (Markowski, 1936). They found heavy infections (environ 80%) of *H. stagnalis* at Canet, but were unable to find infected fishes in the area. The species, assigned to *B. cingulata* by Chabaud and Biguet (1955) was recognized on biological grounds as a new species, *Bunocotyle meridionalis.* They observed that life-cycles in the hemiurid trematodes may be extraordinarily varied, with one, two, three or four hosts. In the genus *Bunocotyle, B. progenetica* becomes mature in the mollusk; *B. meridionalis* becomes mature in the second intermediate host, the copepod; while *B. cingulata* matures in the piscine host. There is the possibility of a four-host-cycle when the copepod host is eaten by small fishes that serve as transport or paratenic hosts. Rebecq (1964) found *B. meridionalis* in *Hydrobia ventrosa* and *Hydrobia acuta* taken in the Camargue, a part of the Rhone delta near Marseille. The gravid metacercariae were found in *Calanipeda aquae-dulcis* (= *Popella guernei*).

The postulate by Sinclair *et al.* (1972) recalls the report by Dollfus (1950b) in which he noted that Looss, Nicoll, Szidat, and other authors had observed young sterrhurine worms encysted in the peritoneum and other organs of various teleost fishes. The significance of these findings is yet obscure and the tendency for larval stages of hemiurid trematodes to become sexually mature, renders interpretation of life-cycles very tenuous. The cystophorous cercariae are regularly eaten by copepods, and these in turn by small fishes where they may become mature in the stomach. Whether or not the mature worms, encysted in small fishes, constitute a stage in a normal life-cycle is questionable. It appears more likely that they are stray individuals in abnormal hosts, which have abandoned the stomach and are destined to perish and become encapsulated in connective tissue.

In the hemiurid trematodes the eggs are small, very numerous, embryonated when passed, and the miracidia emerge only after ingestion by a suitable molluscan host. These features suggest a high larval mortality and great hazards in the completion of the life-cycle. The miracidia of the hemiurid, azygiid and didymozoid species are unique and very similar. All are aciliate, provided with an anterior circle of spines, and the surface of the body bears bristles, often disposed in radial plates at the anterior and sometimes also at the posterior end of the larva. The cercariae develop in rediae; they lack penetration and cystogenous glands and develop into the cystophorous stage which is characteristic for hemiurid trematodes. Typically they are eaten by copepods and the metacercariae occur as unencysted larvae in the hemocoele of the crustacean or other planktonic invertebrates that feed on copepods. Sexually mature worms occur in the stomach or in the body cavities of fishes or they may be encysted in the tissues, often in a dead or disintegrating condition. The striking similarity of the larval stages, and the fact that they are peculiar to the hemiurid trematodes, portends genetic homogeneity and despite adult adaptations to different situations, the thesis of Odhner and Fuhrmann that the Azygiidae, Hemiuridae and Didymozoidae are closely related is probably correct.

SUMMARY

Dinurus pinguis Linton, 1940 was described from a large collection of small trematodes, assembled during the previous forty years. Many of them had been assigned to other, different, species in earlier publications. There was no type-specimen but the account was based primarily on material from *Menidia menidia*. The specific diagnosis was indefinite, imprecise, and so general that it might include members of more than one genus. *Dinurus pinguis* was transferred by Manter (1947) to the genus *Tubulovesicula* Yamaguti, 1934. The species, as restricted, is common in *Menidia menida* at Woods Hole, Massachusetts and is redescribed from juvenile and adult specimens.

Sinclair, Smith and Sullivan (1972) suppressed *Stomachicola magnus* (Manter, 1931) Manter, 1947 as a synonym of *Stomachicola rubeus* (Linton, 1910) Manter, 1947 and predicated that *Tubulovesicula pinguis* is merely a stage in the life-cycle of *S. rubeus*. The identity of *S. rubeus* and *S. magnus* may be accepted, but the proposal that *T. pinguis* may develop into *S. rubeus* is rejected.

The collection, organization and integration of information on the genetic relations and systematics of the hemiurid trematodes discloses different interpretations and evaluations of taxonomic features. The present status is reviewed. The divergence of opinion results in large measure from lack of knowledge of life-cycles and developmental stages of marine species. Information from freshwater species, although limited, is significant and helpful. Homologous features, common to all hemiurid species, include the nature of the eggs, the peculiar bristle-bearing miracidia, the presence of rediae in the life-cycle, the characteristic cystophorous cercariae that are eaten by small crustaceans, and the unencysted metacercariae in various planktonic invertebrates. These striking features, shared by all members, portray a common genetic constitution and support the postulate of Odhner (1905), Fuhrmann (1928) and others that the Azygiidae, Hemiuridae and Didymozoidae are members of a common superfamily.

LITERATURE CITED

Anderson, M. G., and F. M. Anderson, 1963. Life history of *Proterometra* Anderson, 1962. *J. Parasitol.*, **49**: 275–280.

Anderson, M. G., and F. M. Anderson, 1967. The life histories of *Proterometra albacaudata* and *Proterometra septimae* spp. n. (Trematoda: Azygiidae) and a redescription of *Proterometra catenaria* Smith, 1934. *J. Parasitol.*, **53**: 31–37.

Baer, J. G., and C. Joyeux, 1961. Classe des Trématodes. Pages 561–692 in P. P. Grassé, Ed., *Traité de Zoologie,* vol. 4. Masson et Cie, Paris.

Chabaud, A. G., and J. Biguet, 1955. Étude d'un trématode hemiuroide à métacercaire progénétique. *Ann. Parasitol.,* **29**: 527–545.

Chabaud, A. G., and Alice Buttner, 1959. Note complémentaire sur le *Bunocotyle* (Trématode Hemiuroide) de l'Étang du Canet. *Vie Milieu,* **10**: 204–206.

Ching, Hilda L., 1960. Studies on three hemiuroid cercariae from Friday Harbor, Washington. *J. Parasitol.,* **46**: 663–670.

Creutzburg, N., 1890. Untersuchungen über den Bau und die Entwickelung von *Distomum ovocaudatum,* Vulpian. Dissertation, Leipzig-Reudnitz, 32 pp.

Dawes, B., 1949. *The Trematoda.* Cambridge University Press, Cambridge, 644 pp.

Dickerman, E. E., 1934. Studies on the trematode family Azygiidae. I. The morphology and life cycle of *Proterometra macrostoma* Horsfall. *Trans. Amer. Microscop. Soc.,* **53**: 8–21.

Dickerman, E. E., 1946. Studies on the trematode family Azygiidae. III. The morphology and life cycle of *Proterometra sagittaria* n. sp. *Trans. Amer. Microscop. Soc.,* **65**: 37–44.

Dollfus, R. P., 1923. Remarques sur le cycle évolutif des Hémiurides. Note préliminaire. *Ann. Parasitol.,* **1**: 345–351.

Dollfus, R. P., 1949. Sur une cercaire ophthalmoxiphiocerque, *Cercaria isopori* A. Looss, 1894, et sur la délimitation des Allocreadioidea. *Ann. Parasitol.,* **24**: 424–435.

Dollfus, R. P., 1950a. Hôtes et distribution géographiques des cercaires cystophores. *Ann. Parasitol.,* **25**: 276–296.

Dollfus, R. P., 1950b. Trématodes récoltés au Congo belge par le Professeur Paul Brien (mais-août 1937) *Ann. Mus. Belg. Congo,* **1**: 1–136.

Dollfus, R. P., 1954. Métacercaire progénétique de *Derogenes* (Trematoda: Hemiuroidea) chez un copépode parasite de poisson. *Vie Milieu,* **5**: 565–568.

Dollfus, R. P., 1960a. Critique de récentes innovations apportées à la classification des Accacoeliidae. Observations sur les métacercaires de cette famille. *Ann. Parasitol.,* **35**: 648–671.

Dollfus, R. P., 1960b. Distomes des chaetognathes. Part II. List de récoltes ou d'observations de Hemiuroidea ou de Distomes *incertae sedis* chez des Chaetognathes. *Bull. Inst. Pêches Maritimes du Maroc., No.* **4**: 19–45.

Dubois, G., 1929. Les cercaires de la région de Neuchâtel. *These. Bull. Soc. Neuch. Sci. Nat.,* **53**: 1–177.

Faust, E. C., 1929. *Human Helminthology.* Lea and Febiger, Philadelphia, Pennsylvania.

Fuhrmann, O., 1928. Zweite Klasse des Cladus Plathelminthes. Trematoda. in *Kükenthal-Krumbach,* Eds., *Handbuch der Zoologie,* Vol. 2. Walter de Gruyter & Co., Berlin and Leipzig, 140 pp.

Grabda, E., 1947. Formation et morphologie de la cyste d'une *Nematobothrium sardae* W. G. et G. A. MacCallum, 1916, (Didymozoonidae) parasite des branchies de "la Bonite" (*Sarda sarda* Bloch) provenant de la Mer Noire. *Arch. Hydrobiol. Rybactwa,* **13**: 165–179.

Horsfall, M. W., 1934. Studies on the life history and morphology of the cystocercous cercariae. *Trans. Amer. Microscop. Soc.,* **53**: 311–347.

Hunninen, A. W., and R. M. Cable, 1943. The life history of *Lecithaster confusus* Odhner (Trematoda: Hemiuridae). *J. Parasitol.,* **29**: 71–79.

Ishii, Y., 1935. Studies on the family Didymozoidae (Monticelli, 1888). *Jap. J. Zool.,* **6**: 279–335.

Krull, W. H., 1935. Studies on the life history of *Halipegus occidualis* Stafford, 1905. *Amer. Midland Natur.,* **16**: 129–142.

Krupa, P. L., G. H. Cousineau and A. K. Bal, 1969. Electron microscopy of the excretory vesicle of a trematode cercaria. *J. Parasitol.,* **55**: 985–992.

La Rue, G. R., 1957. The classification of digenetic trematodes: a review and a new system. *Exp. Parasitol.,* **6**: 306–344.

Lebour, Marie V., 1912. A review of the British marine cercariae. *Parasitology,* **4**: 416–456.

Leuckart, R., 1886. Die Parasiten des Menschen und die von ihnen herrührenden Krankheiten. Pages 1–96 in *Winter'sche Verlagshandlung,* 2 Aufl., Bd. 1, 3 Lief. Leipzig and Heidelberg.

Leuckart, R., 1899. Die Parasiten des Menschen und die von ihnen herrührenden Krankheiten. Pages 97–440 in *Winter'sche Verlagshandlung,* 2 Aufl., Bd. 1, 4 Lief. Leipzig and Heidelberg.

Linton, E., 1898. Notes on trematode parasites of fishes. *Proc. U.S. Nat. Mus.,* **20**: 507–548.

Linton, E., 1901. Parasites of fishes of the Woods Hole region. *Bull. U.S. Fish Comm.,* **19** (1899) : 267–304.

Linton, E., 1905. Parasites of fishes of Beaufort, North Carolina. *Bull. Bur. Fish.,* **24** (1904) : 323–428.

Linton, E., 1910. Helminth fauna of the Dry Tortugas. II. Trematoda. *Carnegie Inst. Washington, Pub. No. 113, Papers Tortugas Lab.* **4** : 11–98.

Linton, E., 1940. Trematodes from fishes mainly from the Woods Hole region, Massachusetts. *Proc. U.S. Nat. Mus.,* **88** : 1–172.

Looss, A., 1894. Distomen unserer Fische und Frösche. Neue Untersuchungen über Bau und Entwickelung des Distomenkörpers. *Biblioth. Zool.,* **16** : 1–296.

Looss, A., 1907. Beiträge zur Systematik der Distomen (Zur Kenntnis der Familie Hemiuridae). *Zool. Jahrb. Syst.,* **26** : 63–180.

Macy, R. H., W. A. Cook and W. R. DeMott, 1960. Studies on the life cycle of *Halipegus occidualis* Stafford, 1905 (Trematoda: Hemiuridae). *Northwest Sci.,* **34** : 1–17.

Manter, H. W., 1931. Some digenetic trematodes of marine fishes of Beaufort, North Carolina. *Parasitol.,* **28** : 396–411.

Manter, H. W., 1947. The digenetic trematodes of marine fishes of Tortugas, Florida. *Amer. Midland Natur.,* **38** : 257–416.

Manter, H. W., 1954. Some digenetic trematodes from fishes of New Zealand. *Trans. Roy. Soc. N. Zl.,* **82** : 475–568.

Markevich, A. P., 1951. *Parasitic Fauna of Freshwater Fish of the Ukranian SSR.* Kiev, 375 pp. (Russian text, English translation by N. Rafael, 1963).

Markowski, S., 1936. Über die Trematodenfauna der baltischen Mollusken aus der Umgebung der Halbinsel Hel. *Bull. Acad. Polon. Sci. Lett. Series B,* **2** : 285–317.

Nasir, P., and M. T. Diaz, 1971. A revision of the genus *Lecithochirium* Lühe, 1901, and redescription of *L. monticelli* (Linton, 1898) Skrjabin and Gushanskaya, 1955. *Riv. Parasitol.,* **32** : 27–36.

Nicoll, W., 1915. The trematode parasites of North Queensland. Parasites of fishes. *Parasitology,* **8** : 22–41.

Nigrelli, R. F., and H. W. Stunkard, 1947. Studies on the genus *Hirudinella,* giant trematodes of scombriform fishes. *Zoologica (New York),* **31** : 185–196.

Odening, K., 1960. Zur Grosseinteilung der digenetischen Trematoden. *Z. Parasitenk.,* **20** : 170–174.

Odhner, T., 1907. Zur Anatomie der Didymozoen: Ein getrenntgeschlechtlicher Trematode mit rudimentären Hermaphroditismus. *Zoologiska Studier tillägrade Prof. Tullberg. Uppsala,* **1907** : 309–342.

Odhner, T., 1911. Zum natürlichen System der digenen Trematoden. IV. Familie Azygiidae n. fam. *Zool. Anz.,* **38** : 513–531.

Overstreet, P. M., 1969. Digenetic trematodes of marine teleost fishes from Biscayne Bay, Florida. *Tulane Stud. Zool. Bot.,* **15** : 119–176.

Poche, F., 1926. Das System der Platodaria. *Arch. Natur.,* **91** (A) : 1–240.

Powell, E. C., 1972. Optical and electron microscope studies on the excretory bladder of the supposed epitheliocystid cercaria of *Ochetosoma aniarum.* *Z. Parasitenk.,* **40** : 19–30.

Rankin, J. S., Jr., 1944. A review of the trematode genus *Halipegus* Looss, 1899, with an account of the life history of *H. amherstensis* n. sp. *Trans. Amer. Microscop. Soc.,* **63** : 149–164.

Rebecq, J., 1964. Recherches systématiques, biologiques et écologiques sur les formes larvaires de quelques Trématodes de Carmargue. *Thèses, Université d'Aix-Marseille,* 222 pp. Centre Régional de Documentation Pédagogique, Aix-en Provence.

Sewell, R. B. S., 1922. Cercariae Indicae. *Ind. J. Med. Res.,* **10** (Suppl) : 1–370.

Sillman, E. I., 1962. The life history of *Azygia longa* (Leidy, 1851) (Trematoda: Digenea), and notes on *A. acuminata* Goldberger, 1911. *Trans. Amer. Microscop. Soc.*, **81**: 43–65.

Sinclair, N. R., F. G. Smith and J. J. Sullivan, 1972. The *Stomachicola rubea: Tubulovesicula pinguis* enigma. *Proc. Helm. Soc. Washington*, **39**: 253–258.

Sinitzin, D. F., 1905. Studies on the life cycle of trematodes. The distomes of the fishes and frogs in the environs of Warsaw. (Russian text) *Mem. Soc. Nat. Varsovie Biol.*, **15**: 1–210.

Skrjabin, K. I., and L. KH. Gushanskaya, 1954. Hemiurata. Pages 227–653 in K. I. Skrjabin and L. KH. Gushanskaya, Eds., *Trematodes of Animals and Man*, Volume 9. In Russian, Moscow.

Skrjabin, K. I., and L. KH. Gushanskaya, 1955. Hemiurata. Pages 467–748 in K. I. Skrjabin and L. KH. Gushanskaya, Eds., *Trematodes of Animals and Man*, Volume 11. In Russian, Moscow.

Skrjabin, K. I., and L. KH. Gushanskaya, 1956. Hemiurata. Pages 667–897 in K. I. Skrjabin and L. KH. Gushanskaya, Eds., *Trematodes of Animals and Man*, Volume 14. In Russian, Moscow.

Skrjabin, K. I., and L. KH. Gushanskaya, 1960. Hemiurata. Pages 531–627 in K. I. Skrjabin and L. KH. Gushanskaya, Eds., *Trematodes of Animals and Man*, Volume 17. In Russian, Moscow.

Smith, Septima, 1934. *Cercaria catenaria* n. sp., a cystocercous cercaria from Florida and its development into *Proterometra catenaria* n. sp. *J. Alabama Acad. Sci.*, **6**: 16–17.

Smith, Septima, 1936. Life cycle studies of *Cercaria hodgesiana* and *Cercaria melanophora*. *J. Alabama Acad. Sci.*, **8**: 30–32.

Sogandares-Bernal, F., 1959. Digenetic trematodes of marine fishes from the Gulf of Panama and Bimini, British West Indies. *Tulane Stud. Zool.*, **7**: 69–117.

Stunkard, H. W., 1930. The life-history of *Cryptocotyle lingua* (Creplin), with notes on the physiology of the metacercaria. *J. Morphol. Physiol.*, **50**: 143–191.

Stunkard, H. W., 1956. The morphology and life-history of the digenetic trematode, *Azygia sebago* Ward, 1910. *Biol. Bull.*, **111**: 248–268.

Stunkard, H. W., 1963. Systematics, taxonomy, and nomenclature of the Trematoda. *Quart. Rev. Biol.*, **38**: 221–233.

Szidat, L., 1932. Ueber cysticerke Riesencercarien, insbesondere *Cercaria mirabilis* M. Braun und *Cercaria splendens* n. sp., und ihre Entwicklung im Magen von Raubfischen zu Trematoden der Gattung *Azygia* Looss. *Z. Parasitenk.*, **4**: 477–505.

Szidat, L., 1956. Ueber den Entwicklungszyklus mit progenetischen Larvenstadien (Cercariaeen) von *Genarchella genarchella* Travassos, 1928 (Trematoda: Hemiuridae) und die Möglichkeit einer hormonalan Beeinflussung der Parasiten durch ihre Wirtstiere. *Tropenmed. Parasitol.*, **7**: 132–141.

Szidat, L., V. Angelescu and E. Siccardi, 1950. *Dinurus breviductus* Looss, 1907 (Trematoda, Fam. Hemiuridae) agent causal de la "Enfermedad de las manchas negras" de *Clupea melanostoma* Eig. 1907 del Rio de la Plata. *Commun. Instit. Nac. Invest. Sci. Nat.*, **1**: 3–27.

Tang, C. C., 1951. Contribution to the knowledge of the helminth fauna of Fukien. Part 3. Notes on *Genarchopsis chinensis* n. sp., its life history and morphology. *Peking Natur. Hist. Bull.*, **19**: 217–223.

Thomas, L. J., 1939. Life cycle of a fluke, *Halipegus eccentricus* n. sp., found in the ears of frogs. *J. Parasitol.*, **25**: 207–221.

Travassos, L., J. F. Teixeira de Freitas and Anna Kohn, 1969. Trematódeos do Brasil. *Mem. Instit. Oswaldo Cruz*, **67**: 1–886.

Wootton, D. M., 1957. Notes on the life cycle of *Azygia acuminata* Goldberger, 1911 (Azygiidae-Trematoda). *Biol. Bull.*, **113**: 488–498.

Yamaguti, S., 1934. Studies on the helminth fauna of Japan. Part 2. Trematodes of fishes. I. *Jap. J. Zool.*, **5**: 249–541.

Yamaguti, S., 1954. *Systema Helminthum. Part I. Digenetic Trematodes of Fishes.* Tokyo, Japan, 405 pp.

Yamaguti, S., 1958. *Systema Helminthum. The Digenetic Trematodes of Vertebrates.* Interscience Publ., New York, 2 vol.

Yamaguti, S., 1971. *Synopsis of Digenetic Trematodes of Vertebrates.* Keigaku Publ. Co., Ltd., Tokyo, Japan, 2 vol.

Reference: *Biol. Bull.*, **145**: 627–641. (December, 1973)

POLYMORPHIC TERMINATION OF DIAPAUSE BY CECROPIA: GENETIC AND GEOGRAPHICAL ASPECTS

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The Cecropia moth, *Hyalophora cecropia* (L.) (Lepidoptera: Saturniidae), occurs over most of the United States and southern Canada east of the Rocky Mountains. It is apparently entirely univoltine, although Grote (1878) claimed that it is double-brooded in the south. The winter is spent as a diapausing pupa in a cocoon firmly attached to the food plant or a nearby shrub. The adults, which emerge in spring or early summer, do not feed and have an average life span of only about ten days (Rau and Rau, 1914). The larvae, which are polyphagous and feed on a variety of woody plant species, spin and pupate in late summer or early fall (Waldbauer and Sternburg, 1967; Scarbrough, 1970 and Ferguson, 1972).

Diapause in Cecropia has been thought to be obligatory (Williams, 1956), but Mansingh and Smallman (1966, 1967) reported that it can be prevented by exposing the larvae to a long photophase. They did not report the temperature at which the larvae were reared, unfortunate in view of the modifying effect of temperature on critical daylength which has been reported for other species (Danilevskii, 1965). Williams (1956) found that diapause could be terminated by prolonged exposure to low temperatures—for example, 10 weeks at 6° C. Mansingh and Smallman (1966, 1967) reported that diapause could also be terminated in unchilled pupae by long photophase, but thought that a long photophase had no effect on chilled pupae.

In an earlier paper (Sternburg and Waldbauer, 1969) we established that from 1966 to 1968 the emergence of adult Cecropia was bimodal under natural conditions in the vicinity of Urbana, Illinois. Data for three more years confirm our earlier findings and are presented below. Thus, from 1966 to 1971 from 4.7% to 15.6% of the adults emerged from over-wintering pupae during late May and the first few days of June (Group I) while the remainder emerged during the second half of June and the first few days of July (Group II). It seems safe to conclude that the emergence of adult Cecropia is consistently bimodal—at least in the vicinity of Urbana.

We do not know how widely it occurs over the range of the species, but the bimodal emergence of Cecropia is clearly not just a local phenomenon. Data presented by Marsh (1941) indicate a bimodal emergence in Chicago, Illinois in 1933. Dr. A. M. Young (Department of Biology, Lawrence University, personal communication) found the emergence to be bimodal in Chicago in 1968, and we (see below) found it to be bimodal there in 1970. Rau and Rau (1912, 1914) presented data which show clearly that the emergence of Cecropia was bimodal in St. Louis, Missouri in 1910, 1911 and 1913. Data from other parts of the range would be of great interest but are not available.

A comparable bimodality has not been demonstrated by investigators who worked extensively with Cecropia under laboratory conditions (Williams, 1956; Mansingh and Smallman, 1966 and 1967). The reason is unknown. Perhaps they used populations which are not polymorphic for diapause termination—if such populations exist—or perhaps the polymorphism was not expressed under the conditions they used in the laboratory. Williams (1956, Fig. 3) did report a bimodal emergence which extended over a twenty-four week period and was obviously the result of insufficient chilling. We have made similar observations. If wild local pupae are brought into the laboratory at the beginning of December some adults emerge from them two to three months later while most of the remaining surviving adults emerge nine to twelve months later. This appears to be a different phenomenon than the bimodal emergence which under natural conditions extends over a period of only about eight weeks. Williams (1956, Table III) noted two instances of bimodality which might be comparable to our observations, but whether or not they actually are comparable cannot be determined because he presented only the data derived from the primary mode.

Bimodality is not brought about just by an intrinsic difference in the rates at which adult development proceeds in the two groups. Observations made at regular intervals in an outdoor insectary at Urbana in 1968 showed that the median date on which development was initiated (as signalled by the retraction of the epidermis in the area of the legs) was April 1 for Group I but June 3 for Group II. The median emergence dates were May 31 for Group I and June 26 for Group II (Willis, Waldbauer and Sternburg, 1974). Thus, Group II began to develop 63 days later than Group I although the interval between the median emergence dates was only 26 days. The discrepancy is due to the fact that Group I initiated development while the weather was still cool and, therefore, required a mean of about 59 days to complete development, while Group II, which initiated development when the weather was much warmer, required a mean of only about 25 days to complete development.

The extent to which one may validly extrapolate what happens under natural conditions from the results of the published laboratory studies of diapause termination in Cecropia is by no means obvious. Nevertheless, the results obtained by Williams (1956) have often led to the supposition that under natural conditions winter chilling breaks diapause in all pupae, and that all pupae begin to develop as soon as it becomes warm enough, synchronization of individuals with each other occurring because all develop at more or less the same temperature and, therefore, at more or less the same rate. Something like this does seem to be true of Group I, but it is definitely not true of Group II. Group II pupae are capable of deferring the initiation of development for over two months under conditions which permit the initiation and completion of development in Group I pupae. Obviously there are unexpected complexities involved in the resumption of development by Cecropia pupae, and, as will be discussed below, these complexities permit a precise and subtle adjustment to climatic and ecological conditions.

We have not made an exhaustive search of the literature for cases of polymorphic termination, but a careful scrutiny of several of the more recent reviews (deWilde, 1962; Danilevskii, 1965; Beck, 1968; Danilevskii, Goryshin and Tyshchenko, 1970) suggests that the polymorphic termination of diapause is either an infrequent phenomenon or that it tends to go unrecognized. Danilevskii (1965, page 203) cites a report that the emergence of the cotton bollworm (*Chloridea obsoleta*) from overwintering pupae is bimodal in the Caucasus. The emergence of tobacco hornworms (*Manduca sexta*) from overwintering pupae is bimodal in North Carolina

(Rabb, 1966). It has long been known that the emergence of the zebra swallowtail (*Eurytides marcellus*) from overwintering pupae is bimodal, and that in this case the dimorphism involves color and form as well as physiology (Scudder, 1889, pages 1273–1278).

We have used the phrases "resumption of development" and "termination of diapause" as if they are interchangeable. This is the common usage and is consistent with the terminology proposed by Mansingh (1971). However, it should be recognized that this usage tends to obscure the complexity of diapause termination. For example, Mansingh (1971) recognized that insects which are "in diapause" may be in either the "refractory phase" or the "activated phase." Thus, insects which have received insufficient chilling are not competent to resume development, and would be considered refractory, while insects which have received sufficient chilling begin to develop soon after they are warmed up and would be considered activated.

MATERIALS AND METHODS

Collecting cocoons

The cocoons which will henceforth be referred to simply as the "Urbana cocoons" were collected by the authors from the native wild population in the vicinity of Urbana, Illinois (approximately 40°6′ north latitude). Approximately 95% were collected from trees and shrubs in the twin cities of Champaign and Urbana; the remainder were taken in similar situations in the surrounding countryside or nearby small towns. Further details were given by Waldbauer and Sternburg (1967) and by Sternburg and Waldbauer (1969).

In 1967 wild cocoons were collected by Dr. Allen M. Young in Chicago, about 120 miles to the north of Urbana. About one-third were taken from willows along the Illinois Central Railroad tracks about one mile south of the intersection of Michigan and Madison Avenues (approximately 41°51′ north latitude); about one-half were taken along the same tracks about ten miles to the south; the remainder came from various localities within the Chicago city limits. These cocoons were moved to Urbana on December 19, 1967. On December 15, 1969 the authors, Dr. Aubrey Scarbrough and Dr. Young made another collection of cocoons from the sites along the railroad tracks mentioned above. The cocoons were taken to Urbana that day, but the next day one-half of them plus a number of cocoons collected in Urbana were transferred to the Morton Arboretum at Lisle, Illinois (approximately 41°47′ north latitude) about twenty-five miles west of the intersection of Michigan and Madison.

Rearing

Females were mated either by placing them in a cage with a male or by placing them outdoors in a flight trap (Sternburg and Waldbauer, 1969) where they mated with a wild male. The latter technique was used when Group I males were on the wing, and only in the selection of early emerging strains from the local population. Inseminated females, confined in large brown paper bags in the laboratory, fastened about 300 eggs to the sides of the bag in five or six days. When the eggs were nearly ready to hatch the bags were ripped open and placed

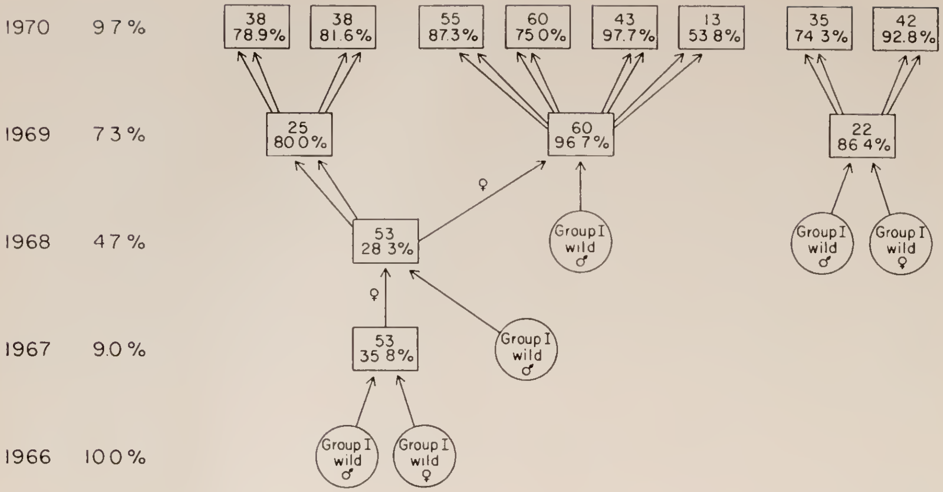

FIGURE 1. The descent of the eight groups of Cecropia siblings, line selected from the Urbana population for emergence with Group I, which emerged as adults in 1970. On the left are the year of emergence and the percentage of the wild population which emerged with Group I in each year. A circle indicates a wild parent, a box the progeny resulting from a controlled mating. The upper figure in each box shows total progeny reared; the lower shows the percentage which emerged with Group I. Arrows show parentage; sex is indicated only where necessary.

under large nylon mesh sleeves (Telfer, 1967) which covered entire apple trees (*Malus pumila*) and were securely tied shut at the base of the trunk. The trees were usually almost defoliated by the time the larvae became third instars; the larvae were then moved to fresh trees—about thirty to a net—where they usually managed to complete their growth without running short of food. In late summer or early fall the nets were removed and the cocoons were harvested.

Maintenance of pupae

Cocoons were kept in screened cages in a screened outdoor insectary at either Urbana or the Morton Arboretum, and were exposed only to natural photoperiods and temperatures. From late April to mid-July the cages were examined daily for newly emerged adults. The date of emergence and sex were noted except at the Morton Arboretum where sex was not noted. Reared animals were indoors only when mating and/or ovipositing. Wild or reared cocoons were sometimes brought into the laboratory for a few hours for counting and sorting.

The daily mean temperatures indicated on Figures 2–4 for Urbana were provided by the Illinois State Water Survey. Daily means for the Morton Arboretum (Fig. 3) were calculated from daily maximum and minimum temperatures provided by the Environmental Data Service of the U. S. Department of Commerce.

Selection of early-emerging strains

Beginning in 1966 we made line selections for early emergence (Group I) from the population native to the Urbana area. In every instance the group affiliation of individuals was determined by reference to the seasonal pattern of emergence from a group of locally collected wild pupae held for the winter in the same insectary. From 1966 to 1968 the emergence pattern was further confirmed by using traps baited with virgin females to catch males from the remaining local wild population (Sternburg and Waldbauer, 1969). We made frequent outcrosses because inbreeding led to a disastrous reduction in viability. Five females which were the F_1 progeny of a wild pair and had been mated with their sibs laid a mean of 238 eggs, but only 54.4% of them hatched. Three females, including two from the same group of siblings, mated with wild males laid a mean of 297 eggs, 96.8% of which hatched. Figure 1 shows the pedigrees of all of the groups selected for emergence with Group I which emerged in 1970. All of our line selections were similarly made. We made several matings of Group II adults, but did not make a serious effort to select for emergence with Group II. It might be possible to mate Group I adults with Group II adults if the lives of Group I adults could be prolonged by holding them in a refrigerator. However, we did not attempt this. Mating and rearing were handled as described above. It was usually not possible to rear all of the progeny resulting from a mating. Therefore, when most of the larvae in a group were third instars we discarded the excess, retaining a group approximately typical of the whole with respect to distribution by instar and size.

RESULTS

Selection for emergence with Group I

Table I summarizes the results of all of the controlled matings which we made from 1966 to 1970. An examination of either this table or of Figure 1 leaves no doubt that the bimodal emergence of adult cecropia has a genetic basis. In three years or less we were able to select from the local population strains which averaged over 75% emergence with Group I as compared with the average of about 8% which was typical of the local wild population (Table II).

We made several controlled matings of wild Group II moths in 1966 (Table I), but made no real effort to select for emergence with this group. Our only attempts in this direction (Table I, 1968) led to an apparent decrease in the proportion of adults which emerged with Group II. However, the number of moths involved is small and this result may not be typical.

Even after several years of selection both Groups I and II remained essentially in phase with the corresponding groups of the wild population as indicated by the median emergence dates in Table I. Median dates do not, of course, tell the whole story. However, a closer comparison shows that with either Group I or II the individual emergence dates of the progeny of the various controlled matings listed in Table I generally fell within the range of dates over which the corresponding wild adults emerged. In only 10 out of 32 cases did a few individuals emerge before or after the corresponding group of the wild population, and then the discrepancy was only from one to four days. There was clearly no tendency to produce intermediates. In fact, our data suggest the opposite tendency. From 1967 to 1971 the number of days from the emergence of the last individual of Group I to the emergence of the first individual of Group II of the wild population ranged from 6 to 16 and averaged 10.4. On the other hand, the corresponding figures for

TABLE I

Yearly distribution between the two emergence groups of adult Cecropia line selected from the Urbana population for emergence with either Group I or Group II. The distribution of adults emerging each year from field-collected pupae is given for comparison. "Wild" refers to members of the local wild population which emerged with the indicated group either in the field or in the insectary from pupae collected in the field. "Selection" refers to the F_1 or F_2 progeny resulting from controlled matings

Year of emergence	Type of mating	Total no.		Emerging with Group I			Emerging with Group II		
		Matings	Moths	Mean %	Range in %	Median emergence date	Mean %	Range in %	Median emergence date
1967	(Field collected)*	—	167	9.0	—	May 26	91.0	—	June 20
	Gp I wild × Gp I wild	4	119	23.5	0–35.8	May 26	76.5	64.2–100	June 18
	Gp II wild × Gp II wild	5	78	5.1	0–9.7	May 24	94.9	90.3–100	June 17
1968	(Field collected)*	—	557	4.7	—	May 24	95.3	—	June 20
	Gp I wild × Gp I wild	2	111	4.5	4.2–4.7	May 21	95.5	95.3–95.8	June 20
	Gp I selection × Gp I wild	2	87	21.8	11.8–28.3	May 26	78.2	71.7–88.2	June 22
	Gp I selection × Gp I selection	2	29	17.2	14.3–20.0	May 22	82.8	80.0–85.7	June 23
	Gp II selection × Gp II selection	2	47	14.9	6.7–18.8	May 30	85.1	81.2–93.3	June 19
1969	(Field collected)	—	409	7.3	—	May 24	92.7	—	June 24
	Gp I wild × Gp I wild	1	22	86.4		May 25	13.6		June 17
	Gp I selection × Gp I wild	3	103	89.3	72.7–96.7	May 25	10.7	3.3–27.3	June 17
	Gp I selection × Gp I selection	2	34	64.7	22.2–80.0	May 24	35.3	20.0–77.8	June 26
1970	(Field collected)	—	165	9.7	—	May 19	90.3	—	June 15
	Gp I selection × Gp I selection	8	324	82.7	53.8–97.7	May 14	17.3	2.3–46.2	June 13
1971	(Field collected)	—	270	15.6	—	May 21	84.4	—	June 17
	Gp I selection × Gp I selection	8	98	72.4	23.1–100	May 23	27.6	0–76.9	June 17

* Data from Sternburg and Waldbauer (1969).

TABLE II

Summary of distribution between the two emergence groups of Cecropia adults from wild pupae collected in Urbana or pupae which resulted from controlled matings of Urbana moths, 1967–1971

	No. matings	Emerging with GP I		Emerging with GP II	
		No.	%	No.	%
Field collected (1967–1971)	—	129	8.2	1,439	91.8
Gp I wild × Gp I wild (1967–1969)	7	52	20.6	200	79.4
Gp I selection × Gp I wild (1968–1969)	5	111	58.4	79	41.6
Gp I selection × Gp I selection (1968–1971)	20	366	75.5	119	24.5
Gp II wild × Gp II wild (1967)	5	4	5.1	74	94.9
Gp II selection × Gp II selection (1968)	2	7	14.9	40	85.1

all Group I controlled matings lumped by year from 1967 to 1971 are 7 to 26 days with a mean of 14.4 days. This wider spacing between the two groups is due largely to a tendency for the emergence of Group I progeny of controlled matings to cluster more tightly about the median. Selection for emergence with Group I did not, however, lead to a greater spread between the median emergence dates of Groups I and II. In the wild population the number of days between the median emergence dates of Groups I and II ranged from 25 to 31 and averaged 27.4. For all progeny of Group I controlled matings lumped by year the corresponding figures are 23 to 33 days with a mean of 27.3 days.

Sternburg and Waldbauer (1969) reported that in each group the males emerge earlier than the females, and that Groups I and II are distinguished not only by an interval of no emergence, but also by the alternating emergence of males and females. This held true for the progeny of controlled matings reported on in this paper. We also reported that Group I is characterized by a sex ratio which favors

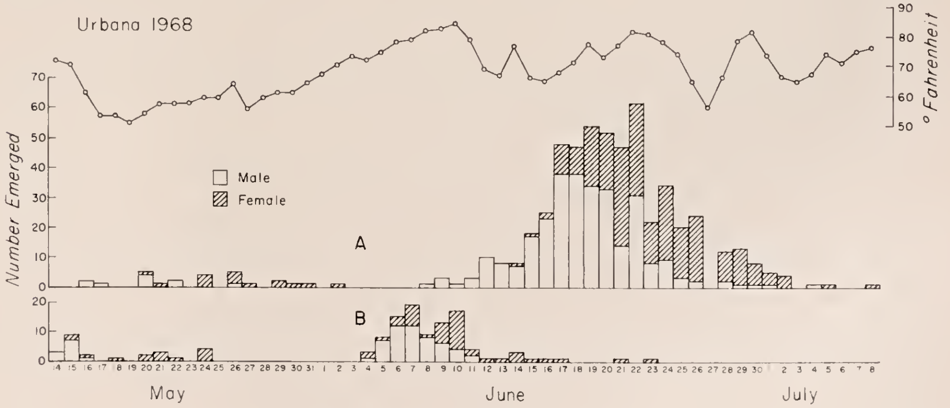

FIGURE 2. Emergence of adult Cecropia at Urbana, Illinois in 1968 from wild cocoons collected in the field and moved into a screened outdoor insectary by late fall; (A) cocoons collected in the vicinity of Urbana; (B) cocoons collected in Chicago. The daily mean temperatures are plotted above the histograms.

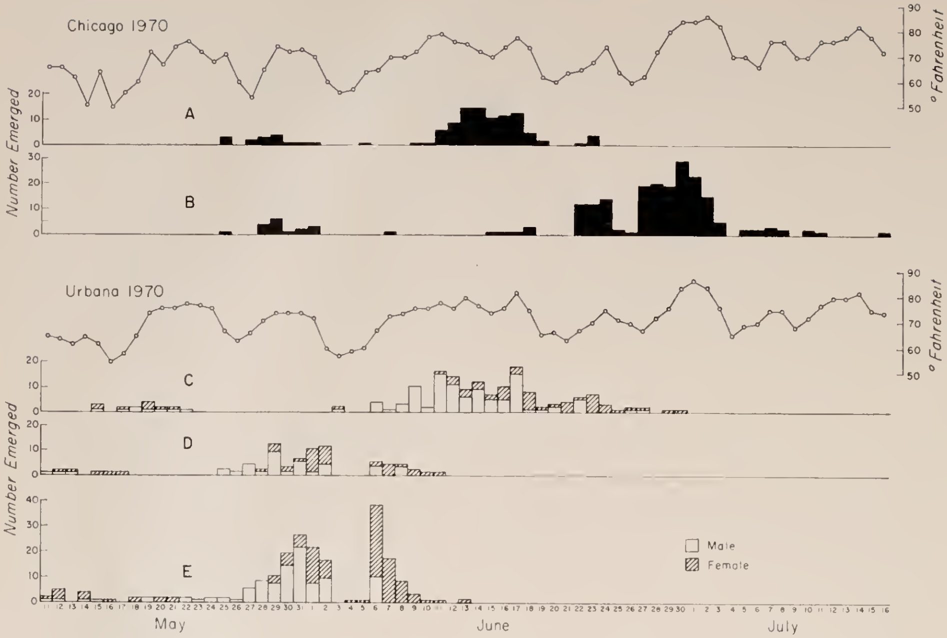

FIGURE 3. Emergence of adult Cecropia in 1970 from cocoons moved into a screened outdoor insectary at either the Morton Arboretum near Chicago (A and B) or at Urbana (C, D and E) by late fall. The origin of the cocoons is as follows: (A) wild, collected in Chicago, (B and C) wild, collected in Urbana, (D) wild, collected in Chicago, and (E) F_2 progeny reared in Urbana from wild parents collected in Chicago in 1967–68. The daily mean temperatures are plotted above the histograms.

females, and Group II by a ratio which favors males. This held only partially true for the progeny of Group I controlled matings. In 1967 and 1968 those which emerged with Group I were consistently mostly females (75% overall), but from 1969 to 1971 the sex ratio consistently approximated 50–50 (overall 48.8% females). On the other hand, the Group II progeny of Group I controlled matings were consistently mostly males (overall 60.9%) from 1967 to 1970 although in 1971 there were only 44.4% males.

Wild adults of both Groups I and II produced some progeny which emerged with Group I and others which emerged with Group II (Tables I and II). A majority of the progeny produced by either group emerged with Group II, but Group I adults usually produced a greater proportion of Group I progeny. Seven matings of wild Group I moths made from 1966 to 1968 produced an average of 20.6% Group I progeny while in the same years an average of only 7.0% of the wild population emerged with Group I. On the other hand, five matings of wild Group II moths made in 1966 produced only 5.1% Group I progeny as compared with the 9% produced by the wild population in the same year.

Latitudinal adaptations in diapause termination

Wild Cecropia collected as pupae in Chicago in the autumns of 1967 and 1969, and transferred to the outdoor insectary at Urbana were partially out of phase

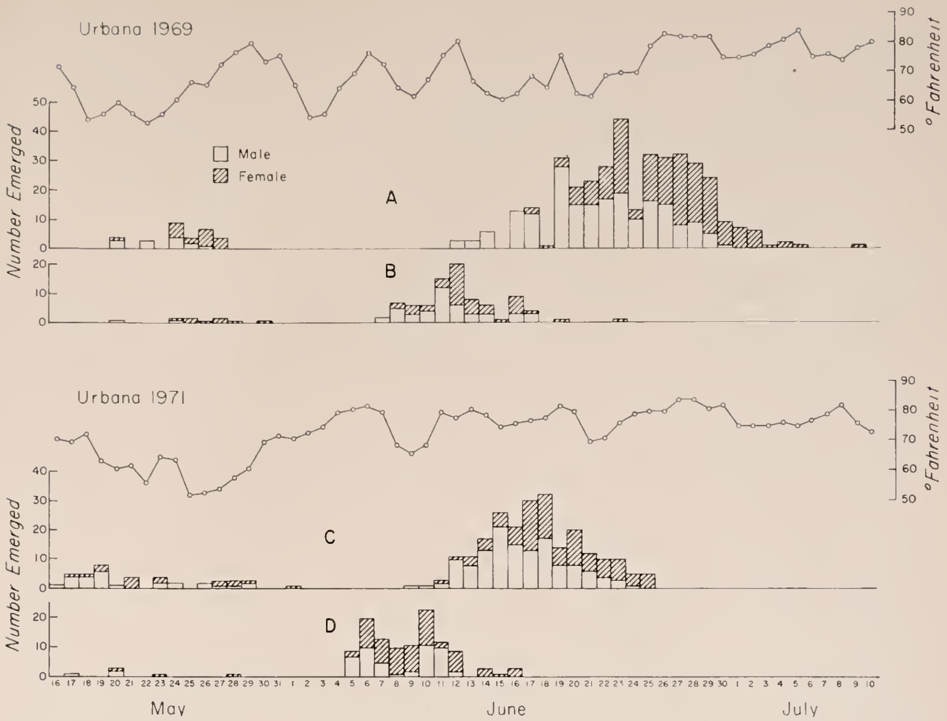

FIGURE 4. Emergence of adult cecropia in 1969 and 1971 from cocoons moved into a screened outdoor insectary at Urbana by late fall. The origin of the cocoons is as follows: (A) wild, collected in Urbana, (B) F_1 progeny reared in Urbana from wild parents collected in Chicago in 1967–68, (C) wild, collected in Urbana, and (D) F_1 progeny reared in Urbana from wild parents collected in Chicago in 1969–70. Mean daily temperatures are plotted above the histograms.

with the local population when the adults emerged the following spring. The reciprocal transfer made in the fall of 1969 revealed that Urbana moths were also partially out of phase with the local population after transfer to the Chicago area early in the pupal stage. In either case the transferred Group I moths were more or less in phase, while the transferred Group II moths were strikingly out of phase with the corresponding groups of the local population (Figs. 2 and 3).

Rearing Cecropia from the Chicago population in Urbana demonstrated that the difference in emergence time between the two populations has a genetic basis. The Group II descendants of Chicago Cecropia remained out of phase with the local Group II population for two generations in one instance (Fig. 3, E) and one generation in two instances (Fig. 4, B and D). Furthermore, after two generations in Urbana the descendants of Chicago Cecropia still emerged in phase with the adults from wild pupae which had been transferred from Chicago to Urbana the previous autumn (Fig. 3, D and E). We did not rear Urbana Cecropia in Chicago.

As pointed out above, Group I Cecropia transferred from Chicago to Urbana or *vice versa* continued to emerge more or less in phase with the local populations

TABLE III

Emergence of adult Cecropia from local cocoons and from cocoons transported between Chicago and Urbana. Unless otherwise indicated cocoons were collected from the wild population in the area of origin and transported no later than December 19. F_1 and F_2 indicate the first and second filial generations reared in Urbana from wild parents collected in Chicago

Origin and ultimate location of pupae	No. adults emerging	% emerging with Gp I	Median emergence date:	
			GP I	GP II
1968				
Urbana in Urbana*	557	4.7	May 24	June 20
Chicago in Urbana	119	17.6	May 16	June 8
1969				
Urbana in Urbana	409	7.3	May 24	June 24
Chicago in Urbana (F_1)	97	11.3	May 25	June 12
1970				
Urbana in Urbana	165	9.7	May 19	June 15
Chicago in Urbana	72	11.1	May 13	June 1
Chicago in Urbana (F_2)	220	9.5	May 14	June 1
Chicago in Chicago	111	14.4	May 29	June 15
Urbana in Chicago	208	8.6	May 29	June 29
1971				
Urbana in Urbana	270	15.6	May 21	June 17
Chicago in Urbana (F_1)	120	5.0	May 20	June 9

* Data from Sternburg and Waldbauer (1969).

(Figs. 2–4). A few of these emergence curves suggest a tendency for Chicago Group I Cecropia in Urbana to emerge slightly earlier than local moths, and the median emergence dates listed in Table III to differ in some cases. However, reference to the curves (Fig. 2, Fig. 3, C and D, C and E) shows that whenever the dates differ the emergence of the two groups nevertheless overlaps to a large extent.

On the other hand, Group II Cecropia transferred either from Chicago to Urbana or *vice versa* were invariably widely out of phase with the local population. Chicago moths reared in Urbana or transferred as pupae emerged from 8 to 14 days *earlier* than the local population (Figs. 2 to 4). Conversely, the Group II Urbana moths in Chicago emerged 14 days *later* than the local population (Fig. 3, A and B).

It should be noted that in any case—whether a transfer is involved or not—the emergence of Group II follows sooner after the emergence of Group I in the Chicago population than it does in the Urbana population. The time which elapses from the median emergence date of Group I to the median emergence date of Group II varies somewhat from year to year, but remains more or less constant for each population, averaging 19 days for the Chicago population and 28 days for the Urbana population. The difference was invariably greater in the Urbana population (Table IV). It is, of course, adaptive for the difference in emergence time between the two groups to decrease to the north since less delay is possible where the season is shorter.

The reciprocal transfers of 1969-70 showed that, with respect to the population left behind, being moved to the north delays the emergence of both Groups I and

II while being moved to the south accelerates the emergence of both Groups I and II (Fig. 3, A to D, Table III). This is, of course, the expected result. From Urbana pupae in Chicago, Group I and II moths emerged respectively 10 and 14 days *later* than their counterparts in Urbana. From Chicago pupae in Urbana, Group I and II moths emerged respectively 16 and 14 days *earlier* than their counterparts in Chicago.

Discussion

The polymorphic termination of diapause by Cecropia, considered either from the ecological or the physiological point of view, appears to be of considerable importance to the species. The difference in the emergence times of the two groups, consistently almost a month in the Urbana area, is equal to at least one-fifth of the growing season available to Cecropia at this latitude. It is almost inconceivable that such a large difference does not have profound ecological implications for the species. From a physiological point of view, the consistent difference of over two months in the onset of development by the two groups cannot be considered to be trivial.

We believe that Cecropia's dimorphic termination of diapause is adaptive. The strategy involved is perhaps best expressed by the metaphor which warns against putting all of the eggs in one basket. Cecropia's dimorphism avoids the placing of all of the progeny of a pair in one "temporal basket" in the following growing season at the critical period encompassing adult development, emergence and reproduction. Partitioning the progeny between an early and a late emergence group might allow at least some of the progeny of a pair to escape various detrimental factors which may occur at different times in the growing season, but do not occur in every year or vary in severity from year to year, such as some variations of the

Table IV

Days between median emergence dates of adult Cecropia from local cocoons and cocoons transferred between Chicago and Urbana. See Table III for further information

Origin, ultimate location of pupae and year	Days
Chicago in Chicago—1970	17
Chicago in Urbana—1968	23
Chicago in Urbana—1970	19
Chicago (F_1) in Urbana—1969	18
Chicago (F_1) in Urbana—1970	20
Chicago (F_2) in Urbana—1970	18
Mean	19.2
Urbana in Urbana—1966	26
Urbana in Urbana—1967	26
Urbana in Urbana—1968	27
Urbana in Urbana—1969	31
Urbana in Urbana—1970	27
Urbana in Urbana—1971	27
Urbana in Chicago—1970	31
Mean	27.9

weather. Such detrimental factors might affect the developing pharate adults, the emerged adults or their progeny. For example, Group II and their progeny might escape the effects of unseasonably cold weather early in the season which might be disastrous for Group I, killing either the Group I adults themselves or their progeny. On the other hand, in another year the progeny of Group I moths might complete their larval development early enough to avoid a late summer drought which might be disastrous for the progeny of Group II. This strategy could also be selected for by other factors; for example, pressure from a parasitoid whose population fluctuates greatly from year to year and which attacks the progeny of only one of the emergence groups. The strategy of dimorphic diapause termination still allows exploitation of the entire growing season in a year in which the detrimental factors do not occur. The relevant factors may be difficult to identify if they are of relatively infrequent occurrence.

The proportion of individuals which emerge with the two groups varies from year to year. In Urbana from 1967 to 1971 Group I varied from 4.7% to 15.6% of the total (Table I). In St. Louis, Missouri Group I varied between 18% and 58% of the total from 1910 to 1913 (Rau and Rau, 1912 and 1914). The large differences in St. Louis suggest differential mortality, and are probably evidence of major shifts in the balance of factors which determine the relative survival of members of the two emergence groups. Smaller changes, such as those encountered at Urbana, suggest that selection for the polymorphic termination of diapause operates at at least a low level in every year. On the other hand, factors other than differential survival may in part account for these variations. Although polymorphic termination has a genetic basis, environmental factors might affect its phenotypic expression. In other words, the proportion of the population which could potentially emerge with Group I might be greater than the proportion which actually does emerge with Group I—the difference varying with environmental influences. This might explain what happened in 1968 when in Chicago almost 50% of a group of locally collected pupae produced Group I moths (A. M. Young, personal communication) while the other half of the same collection, moved to Urbana before winter, produced only 17.6% Group I moths (Fig. 2, Table III). The total mortality in this collection was far from sufficient to account for this difference.

It would be interesting to know how the mean proportions of the two emergence groups vary geographically. There might, for example, be a systematic variation from north to south.

The establishment of a genetic basis for bimodality is in agreement with our earlier suggestion (Sternburg and Waldbauer, 1969) that emergence with Group I or Group II is an expression of an inherent property of the individual. In the earlier paper we arrived at this conclusion by comparing curves obtained in two ways. The first were curves of adult emergence from cocoons collected locally early in the fall and kept during the winter and spring in an insectary under conditions common to all. The second were flight curves obtained by trapping males which emerged from that portion of the local population of pupae which remained in the field in a variety of microenvironments. The fact that the two sets of curves were in phase and included similar proportions of Group I and Group II emergers indicated that bimodality could not be ascribed simply to differences in the microenvironments in which the cocoons spent the winter and spring.

It has been suggested that the emergence groups of Cecropia might constitute two sibling species which are morphologically indistinguishable (Ferguson, 1972, page 251). This is obviously impossible. Although the two groups are well separated in time they are clearly not reproductively isolated from each other since the adults of both groups produce a mixed lot of pogeny, some of which emerge with Group I and some of which emerge with Group II.

We originally proposed (Sternburg and Waldbauer, 1969) that the bimodal emergence of Cecropia is actually the expression of overlapping latitudinal adaptations of the diapause terminating mechanism. This explanation now seems altogether improbable in light of the results given above (Fig. 3, Table III).

There have been many reports of intraspecific geographical adaptations of the diapause-controlling mechanisms of various species, but most of these have been of changes in the critical daylength required for the *induction* of diapause (Danilevskii, 1965; Danilevskii *et al.*, 1970). There are few reports of intraspecific geographical adaptations of the mechanisms which *terminate* diapause. Indeed, Danilevskii *et al.* (1970) offered the generalization that when diapausing stages are moved to the north of the range they resume development in the spring simultaneously with the native population. The implication is, of course, that the environmental prerequisites for the resumption of development are identical for the two groups, and that there has, therefore, been no geographical adaptation.

Cecropia's response is thus unexpectedly complex. When diapausing Group I pupae were moved from Urbana north to Chicago or *vice versa* the adults did emerge more or less synchronously with the native adults. Obviously, Group I exhibits little or no geographic adaptation in diapause termination. Group II pupae, on the other hand, are able to delay the onset of development for a long period of time under conditions which permit Group I pupae to complete their development. Furthermore, the duration of the delay is susceptible to geographic adaptation; as pointed out above, the emergence of Group II follows more quickly after the emergence of Group I in the Chicago population than it does in the Urbana population (Figs. 2, 3, 4, Table IV). It is this difference in the duration of the delay which is largely responsible for the asynchronous emergence of native and transported adults.

As pointed out above, Group I pupae begin to develop in Urbana very early in the spring—at the end of March or the beginning of April. It is likely that in these pupae diapause is terminated simply by chilling, as suggested by Williams (1956), and that adult development begins just as soon as it becomes warm enough. Group II pupae obviously need something more to terminate their diapause. We assume that the latitudinal differences reflect quantitative modifications of the same mechanism which permits Group II pupae to delay the onset of development. We do not believe that qualitatively different mechanisms are involved. It is logically possible that the responsible factor is simply the passage of time. However, it seems more likely that the mechanism requires a response to some environmental factor. This might be a cumulative response or a trigger-like response to a "cue." Photoperiod alone is probably not responsible. If it were, Group II pupae would presumably respond to the lengthening days of spring by initiating development when the photophase becomes sufficiently long. Thus a transfer to the north should result in earlier emergence rather than later emergence as is the case.

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SUMMARY

Hyalophora cecropia (L.) is dimorphic for the termination of diapause at Urbana and Chicago, Illinois, about 40°6′ and 41°51′ north latitude respectively. Under natural conditions the emergence of the adults is clearly bimodal in both places. At Urbana the early emergers (Group I) initiate development at the end of March and emerge during the second half of May; the late emergers (Group II) initiate development at the beginning of June and emerge during the second half of June and the first week of July. Members of either group produce some progeny which emerge with Group I and a majority which emerge with Group II.

The bimodality has a genetic basis. In either two or three generations we were able to line select from the Urbana population strains which averaged over 75% emergence with Group I as compared with the average of about 8% which was typical of the wild population.

Reciprocal transfers in the fall of wild-collected cocoons between Urbana and the Chicago area revealed that Group II exhibits geographic adaptation but that Group I does not. Pupae transferred in either direction produced Group I adults which emerged more or less synchronously with the local wild Group I. On the other hand, in Chicago the median emergence date of Group II Urbana moths was two weeks *later* than the median emergence date of the local Group II; in Urbana the median emergence date of Group II Chicago moths was twelve to fourteen days *earlier* than the median emergence date of the local Group II.

Rearing the progeny of Chicago moths in Urbana established that there is a genetic basis for the difference in emergence time between the Urbana and Chicago populations. Group II of the Urbana-reared F_1 and F_2 descendants of Chicago cecropia continued to emerge earlier than the local Group II. Furthermore, Group II of the F_2 generation emerged synchronously with wild Group II individuals collected in Chicago and transferred to Urbana the previous fall.

Whether a transfer was involved or not, the interval between the emergence of Groups I and II was shorter for the Chicago population than for the Urbana population, the time between median emergence dates averaging nineteen and twenty-eight days, respectivly, for the Chicago and Urbana populations.

LITERATURE CITED

Beck, S. D., 1968. *Insect Photoperiodism.* Academic Press, New York and London, 288 pp.

Danilevskii, A. S., 1965. *Photoperiodism and Seasonal Development of Insects.* [translated from the Russian by J. Johnston] Oliver and Boyd, Edinburgh and London, 283 pp.

DANILEVSKII, A. S., N. I. GORYSHIN AND V. P. TYSHCHENKO, 1970. Biological rhythms in terrestrial arthropods. *Ann. Rev. Ent.*, **15**: 201–244.

FERGUSON, D. C., 1972. Bombycoidea (in part). Pages 155–275 in R. B. Dominick, C. R. Edwards, D. C. Ferguson, J. G. Franclemont, R. W. Hodges and E. G. Munroe, Eds., *The Moths of America North of Mexico.* E. W. Classey, London.

GROTE, A. R., 1878. In the Minutes of the Annual Meeting of the Entomological Club of the AAAS. *Can. Entomol.*, **10**: 176.

MANSINGH, A., 1971. Physiological classification of dormancies in insects. *Can. Entomol.*, **103**: 983–1009.

MANSINGH, A., AND B. N. SMALLMAN, 1966. Photoperiod control of an "obligatory" pupal diapause. *Can. Entomol.*, **98**: 613–616.

MANSINGH, A., AND B. N. SMALLMAN, 1967. Effect of photoperiod on the incidence and physiology of diapause in two saturniids. *J. Insect Physiol.*, **13**: 1147–1162.

MARSH, F. L., 1941. A few life-history details of *Samia cecropia* within the southwestern limits of Chicago. *Ecology*, **22**: 331–337.

RABB, R. L., 1966. Diapause in *Protoparce sexta* (Lepidoptera: Sphingidae). *Ann. Ent. Soc. Amer.*, **59**: 160–165.

RAU, P., AND N. RAU, 1912. Longevity in saturniid moths: an experimental study. *J. Exp. Biol.*, **12**: 179–204.

RAU, P., AND N. RAU, 1914. Longevity in saturniid moths and its relation to the function of reproduction. *Trans. Acad. Sci. St. Louis*, **23**: 1–78.

SCARBROUGH, A. G., 1970. The occurrence of *Hyalophora cecropia* (L.) as related to urbanization. *Ph.D. thesis, University of Illinois*, Urbana, Illinois, 212 pp.

SCUDDER, S. H., 1889. *The Butterflies of the Eastern United States and Canada.* S. H. Scudder, Cambridge, Massachusetts, Vol. II.

STERNBURG, J. G., AND G. P. WALDBAUER, 1969. Biomodal emergence of adult cecropia moths under natural conditions. *Ann. Ent. Soc. Amer.*, **62**: 1422–1429.

TELFER, W. H., 1967. Cecropia. Pages 173–182 in F. H. Wilt and N. K. Wessels, Eds., *Methods in Developmental Biology.* T. Y. Crowell Co., New York.

WALDBAUER, G. P., AND J. G. STERNBURG, 1967. Host plants and the locations of the baggy and compact cocoons of *Hyalophora cecropia* (Lepidoptera: Saturniidae). *Ann. Ent. Soc. Amer.*, **60**: 97–101.

WILDE, J. DE, 1962. Photoperiodism in insects and mites. *Ann. Rev. Ent.*, **7**: 1–26.

WILLIAMS, C. M., 1956. Physiology of insect diapause. X. An endocrine mechanism for the influence of temperature on the diapausing pupae of the cecropia silkworm. *Biol. Bull.*, **110**: 201–218.

WILLIS, J. H., G. P. WALDBAUER AND J. G. STERNBURG, 1974. The initiation of development by the early and late emerging morphs of cecropia. *Ent. Exp. Appl.*, **17**: in press.

Note added in proof: Mr. C. B. Worth (R.D., Delmont, N.J. 08314) (1973, unpublished manuscript) recently informed us that the emergence pattern of Cecropia is bimodal in Cape May County, New Jersey and that he has demonstrated that early and late emergence have a genetic basis.

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